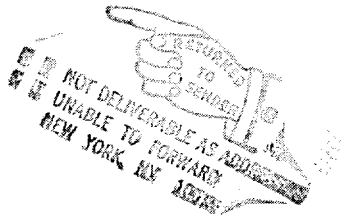
Organization TC1600 Bldg./Room REMSEN
U. S. DEPARTMENT OF COMMERCE
COMMISSIONER FOR PATENTS
P.O. BOX 1450
ALEXANDRIA, VA 22313-1450
IF UNDELIVERABLE RETURN IN TEN DAYS

OFFICIAL BUSINESS

AN EQUAL OPPORTUNITY EMPLOYER



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/051,044	01/14/2002	Mirella Ezban	5994.504-US	1706

7590 03/25/2004

Reza Green, Esq.
Novo Nordisk of North America, Inc.
Suite 6400
405 Lexington Avenue
New York, NY 10174-6401

EXAMINER	
SCHNIZER, HOLLY G	

ART UNIT	PAPER NUMBER
1653	

DATE MAILED: 03/25/2004

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/051,044	EZBAN ET AL.	
	Examiner	Art Unit	
	Holly Schnizer	1653	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *24 May 2002*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *17-23* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *17-23* is/are rejected.

7)☒ Claim(s) *18-20* is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *14 January 2002* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☒ All b)☐ Some * c)☐ None of:

1.☒ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date *1-14-02*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

Status of the Claims

The Preliminary Amendment filed January 14, 2002 has been entered. Claims 1-16 have been cancelled. Therefore, Claims 17-22 are pending and have been considered in this Office Action.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for a patent.
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 17 and 18 are rejected under 35 U.S.C. 102(a) as being anticipated by Taniguchi et al. (Cancer Res. (1998) 58: 4461-4467).

Taniguchi et al. teach that the expression of the urokinase receptor gene is upregulated in response to contacting factor VIIa to human pancreatic cancer cell lines that overexpress Tissue Factor (see abstract). Taniguchi et al. teach that the upregulation of the urokinase receptor gene can be reversed by coincubating the cells with factor VIIa and an anti-TF monoclonal antibody (Fig. 4).

Claims 17-18 and 21-23 are rejected under 35 U.S.C. 102(b) as being anticipated by Pendurthi et al. (Proc. Natl. Acad. Sci. (1997) 94: 12598-12603).

Pendurthi et al. teach that contacting a fibroblast cell line with factor VIIa results in the upregulation of the gene encoding poly (A) polymerase (see abstract). Thus, Pendurthi et al. meets the limitations of Claims 17-18. Pendurthi et al. indicate that 10 nM of factor VIIa was contacted with the fibroblasts (see Figs. 1 and 2) in the method described therein. The method of Pendurthi et al. has the same steps as that of the presently claimed invention and uses the same cell type (fibroblasts) and same concentration of factor VIIa used in the present invention to cause the upregulation of Cyr61 (see example 12 of the present Specification). Thus, the method of Pendurthi et al., having the same steps and components as that of the presently claimed invention, would inherently have the same results (upregulation of Cyr61). Thus, Claims 21-23 are also anticipated by Pendurthi et al.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 17-23 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for a method of increasing expression of at least one gene in a cell line that constitutively expresses tissue factor, wherein the gene is selected from the group consisting of Cyr61, CTFG, dopamine D2 receptor, EST Incyte PD 395116 and P2U nucleotide receptor, does not reasonably provide enablement for a method of regulating the expression of at least one gene in a cell, comprising the step of

contacting said cell with a tissue factor agonist or antagonist, under conditions that result in a measurable change in said expression. More specifically, the specification does not provide enablement for 1) using the claimed method to *down regulate* the expression of a gene in a cell by contacting the cell with a tissue factor agonist or antagonist, 2) using an *antagonist or an agonist other than factor VIIa* to regulate expression of a gene; 3) regulating a gene in a cell other than a *cell line that constitutively expresses tissue factor* or 4) regulating the expression of any gene in any cell using antagonists or agonists of tissue factor. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention commensurate in scope with these claims. Practicing the invention commensurate in scope with the claims would require undue experimentation. Factors to be considered in determining whether undue experimentation is required, are summarized in In re Wands (858 F2d, 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988)). These factors include (1) quantity of experimentation, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims.

Breadth of the Claims:

Claim 17 encompasses a method of regulating the expression of any gene in any cell using any agonist or antagonist of tissue factor. Claim 18 and 19-20 are narrowed from claim 17 to define the agonist or antagonist used in the method but are

still open to the regulation of any gene in any cell. Claims 21-23 are narrowed from claim 17 to define the gene to be regulated but are still open to using any agonist or antagonist of tissue factor and using any cell.

Nature of the Invention:

The nature of the invention involves the identification of several genes, including Cyr61, CTFG, dopamine D2 receptor, EST Incyte PD 395116, and P2U nucleotide receptor, that are upregulated in tissue factor expressing cells that are contacted with factor VIIa, The nature of the invention requires the interaction of tissue factor with its agonist (factor VIIa) for the upregulation of the genes described.

Amount of Direction/Guidance Provided and Presence/Absence of Working Examples:

The present Specification provides guidance and working examples describing the upregulation of genes encoding Cyr61, CTFG, dopamine D2 receptor, EST Incyte PD 395116, the P2U nucleotide receptor, and the urokinase receptor gene. The present specification does not provide any guidance or working examples of regulating gene expression using any antagonists of factor VII or any agonists other than factor VIIa. There is also no guidance or working examples of regulating gene expression in vivo or using cells that do not express tissue factor.

State of the Prior Art/Relative Skill of those in the Art:

As evidenced by Taniguchi et al. and Pendurthi et al. described in the prior art rejections above, those of skill in the art were aware at the time of the present invention that factor VIIa could be used to upregulate gene expression. Taniguchi et al. teaches that contacting factor VIIa with human pancreatic cell lines that overexpress tissue

factor results in increased expression of the gene encoding the urokinase receptor. Pendurthi et al. teach that contacting a fibroblast cell line with factor VIIa results in the upregulation of the gene encoding poly(A) polymerase. However, upregulation of genes by factor VIIa appears to require tissue factor and proteolitically active factor VII (FVIIa) (see Camerer et al. (J. Biol. Chem. (1999) 274(45): 32225-32233 at p. 32231, Col. 2, paragraph 2). Camerer et al. show that while contact of FVIIa to cells expressing tissue factor upregulates egr-1 expression, active site inhibited FVIIa did not induce a response in egr-1 mRNA (see p. 32229, Col. 1, 3rd full paragraph). Pendurthi et al. (J. Biol. Chem. (2000) 275(19): 14632-14641) shows that factor VIIa catalytic activity is required for the induced expression of Cyr61 and that a factor VII modified with D-Phe-L-Phe-L-Arg chloromethyl ketone (active-site inactivated FVIIa) did not induce expression of Cyr61 (see Fig. 6 and p. 14633, Col. 1, "Proteins"). A search of the prior art indicates that the studies of factor VIIa induction of expression have only been in vitro and Camerer et al. indicates that it remains to be seen what the physiological impact of the in vitro observations will be since many physiological pathways seem to be affected by factor VIIa (see Camerer et al. (1999), p. 32232, last paragraph).

Predictability/Unpredictability:

Regulation of gene expression by contacting a protein to a cell involves many different proteins in a signal cascade and is thus very complex. The mechanism of how factor VIIa upregulates the genes is poorly understood. Thus, the predictability of what genes could be regulated, what tissue factor antagonists or agonists could be used to

regulate gene expression, and what cells other than cell lines that constituitively express tissue factor could be used is highly unpredictable.

Quantity of Experimentation:

For the reasons stated above, the quantity of experimentation to practice the claimed invention is considered undue. To practice the instant invention in a manner consistent with the breadth of the claims would not require just a repetition of the work that is described in the instant application but a substantial inventive contribution on the part of a practitioner which would involve the characterization of the mechanism by which factor VIIa, through its contact with tissue factor, upregulates gene expression. It is this additional characterization (that is required to predict with a reasonable expectation of success what tissue factor agonists or antagonists besides factor VIIa could be used in the method and what genes could be regulated by the method) that constitutes undue experimentation. Thus, the full scope of the claims is not considered enabled by the present Specification.

Claim Objections

Claims 18-20 are objected to for using the acronyms, FVII and FVIIa in Claims 18-19. The acronyms are inconsistent with the use of the full name in Claim 19. If acronyms are desired, the examiner suggests printing out the full name in the claim in which it first appears followed by the acronym in parenthesis. For example, Claim 18 could recite "factor VII (FVII), factor VIIa (FVIIa)" in line 2 and then the acronym could

be used in 20. Likewise, Claim 19 should be amended to have the full name of factor VII.

For the reasons stated above, Claim 22 is objected to for the acronym, CTFG. The claim should be amended to include the full name of the gene so that it is clear as to what gene is being referred.

Conclusions

No Claims are allowable.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Holly Schnizer whose telephone number is (571) 272-0958. The examiner can normally be reached on Tuesday, Thursday, and Friday from 8 am to 5:30 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Christopher Low can be reached on (571) 272-0951. The fax phone number for the organization where this application or proceeding is assigned is 703-872-9306.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Holly Schnizer
March 18, 2004

ROBERT A. WAX
PRIMARY EXAMINER

FORM PTO-1449 (Rev. 2-32)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	Atty. Docket No. 5994.504-US	Serial No. ~~To be assigned~~ 10/051,044
	INFORMATION DISCLOSURE STATEMENT BY APPLICANT	Applicant Ezban et al.	
	(Use several sheets if necessary)	Filing Date January 14, 2002	Group ~~To be assigned~~ 1653

U.S. PATENT DOCUMENTS

EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO
HS		WO 97/33995	09/18/97	WIPO	—	—		
HS		WO 99/03498	01/28/99	WIPO	—	—		
HS		WO 99/66031	12/23/99	WIPO	—	—		

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

HS			Sato et al., Thrombosis Haemostasis, Vol. 97, pgs. 1138-1141 (1997)

EXAMINER [signature] DATE CONSIDERED 3-18-04

EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Notice of References Cited	Application/Control No. 10/051,044	Applicant(s)/Patent Under Reexamination EZBAN ET AL.	
	Examiner Holly Schnizer	Art Unit 1653	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Taniguchi et al. Enhanced Expression of Urokinase Receptor Induced through the Tissue Factor-Factor VIIa Pathway in Huma Pancreatic Cancer. (1998) Cancer Res. Vol. 58, pp. 4461-4467.
	V	Pendurthi et al. Binding of Factor VIIa to Tissue Factor Induces Alterations in Gene Expression in Human Fibroblast Cells: Up regulation of Poly(A) polymerase. (1997) Proc. Natl. Acad. Sci. Vol. 94, pp. 12598-12603.
	W	Camerer et al. Coagulation Factors VIIa and Xa Induce Cell Signaling Leading to Up-regulation of the egr-1 Gene. (1999) Vol. 274, No. 45, pp. 32225-32233.
	X	Pendurthi et al. Factor VIIa and Thrombin Induce the Expression of Cyr61 and Connective Tissue Growth Factor (2000) J. Biol Chem. Vol. 275, No. 19, pp. 14632-14641.

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

[CANCER RESEARCH 58, 4461–4467, October 1, 1998]

Enhanced Expression of Urokinase Receptor Induced through the Tissue Factor-Factor VIIa Pathway in Human Pancreatic Cancer[1]

Tadaaki Taniguchi, Ajay K. Kakkar, Edward G. D. Tuddenham, Robin C. N. Williamson, and Nicholas R. Lemoine[2]

Imperial Cancer Research Fund Molecular Oncology Unit [T. T., N. R. L.], Department of Surgery [T. T., A. K. K., R. C. N. W.], and Haemostasis Research Group, Medical Research Council Clinical Sciences Centre [E. G. D. T.], Imperial College School of Medicine, London W12 0NN, United Kingdom

ABSTRACT

Overexpression of tissue factor (TF) is characteristically observed in advanced pancreatic cancer and has been associated with invasion and metastasis. Functional responses of TF activation are here investigated using as a model system the human pancreatic cancer cell lines SW979 (which overexpresses TF) and MIAPaCa2 (which does not express detectable levels). After stimulation of these cell lines with factor VIIa (FVIIa), the only known TF ligand, expression of urokinase receptor (uPAR) gene was up-regulated in SW979 cells in a dose-dependent manner but not in MIAPaCa2 cells. Interestingly, urokinase (uPA) and its specific inhibitor PAI-1 were not up-regulated. Exposure to functionally inactivated FVIIa did not show any effect on uPAR expression on SW979 cells despite binding to TF with higher efficiency. The neutralizing anti-TF antibody 5G9 blocked the FVIIa-induced up-regulation of uPAR completely, whereas hirudin failed to block this up-regulation. Treatment of SW979 cells with Factor Xa did not up-regulate the expression of *uPAR* gene, whereas treatment with FVII induced the same level of enhanced *uPAR* gene expression as that with FVIIa. In the matrigel invasion assay, enhanced invasion of SW979 cell line induced by FVIIa was completely inhibited by anti-TF antibody and α_2-antiplasmin. Moreover, the endogenous levels of *uPAR* gene expression were significantly correlated with the level of *TF* gene expression in 19 human cancer cell lines ($P < 0.05$). These data suggest that up-regulation of uPAR expression by tumor cells leading to tumor invasion is induced through the TF-FVIIa pathway rather than TF-initiated thrombin generation. This is the first report that TF may be one of the key receptors that can up-regulate expression of the plasminogen activator receptor in human cancer cells to enhance tumor invasion and metastasis.

INTRODUCTION

TF[3] is a transmembrane glycoprotein that can activate the coagulation zymogen FVII to the activated form FVIIa. The TF/FVIIa complex initiates the coagulation cascade leading to thrombin generation. TF is also a genuine receptor inducing an intracellular signal upon binding of its specific ligand FVII. It has been reported that TF is highly expressed in cancer cell lines that show enhanced metastatic potential, and it is also involved in the regulation of tumor growth (1, 2). Most colorectal cancer cell lines express TF, and the sublines established from metastatic lesions express higher cellular TF activity than the parental lines (3). Functional inhibition of TF using an anti-TF antibody results in a significant reduction of the number of experimental pulmonary metastases in severe combined immunodeficient mice (1). However, thus far the mechanism of the increased metastatic potential induced by TF expression or activation is still unclear.

TF is a member of the class II cytokine receptor superfamily that includes the IFN α/β and γ receptor (4, 5). The cytoplasmic domain of TF is phosphorylated by a protein kinase C-dependent mechanism in response to phorbol 12-myristate 13-acetate (6). TF mediates a cytosolic Ca^{2+} signal upon interaction with FVIIa in J82 cells (7). Recently, several reports suggested that the binding of FVIIa to cell surface TF may induce transcriptional signaling. The present studies have been carried out to examine the hypothesis that the binding of FVIIa to TF may trigger the selective expression of genes that are involved in the invasive phenotype of cancer cells.

To identify alterations in gene expression in human pancreatic cancer cell lines upon binding of FVIIa to TF, we have studied expression of the components of the plasminogen activator system, which regulates fibrinolysis, by Northern blot analysis. uPA plays an important role in pericellular proteolysis during cell migration and tissue remodeling by physiological activation of plasminogen to plasmin. Binding of uPA to its receptor, uPAR, accelerates uPA activation from an enzymatically inactive proenzyme (pro-uPA). Significantly, overexpression of uPAR increased invasion by facilitating matrix degradation in a human osteosarcoma cell line (8). In human bladder cancer cell lines, both uPAR expression and uPA expression are required for cell invasion (9). The expression of genes of the plasminogen-activator system is differentially regulated by inhibitors of protein synthesis and butyrate (10–12).

In the present study, we found that there was selective overexpression of the uPAR gene induced by FVIIa binding to cell surface TF in human pancreatic cancer cells. Moreover, the endogenous levels of *uPAR* gene expression were correlated with the expression of *TF* gene in 19 human cancer cell lines. We believe that these observations suggest a mechanism by which TF is implicated in tumor invasion and metastasis.

MATERIALS AND METHODS

Materials. FVIIa and FVIIa*i* were provided by Prof. E. G. D. Tuddenham (Medical Research Council Clinical Sciences Center, Imperial College School of Medicine, Hammersmith Hospital, London). FVIIa*i* was derived by the blocking of FVIIa in the active site with D-Phe-L-Phe-L-Arg chloromethyl ketone as described previously (13). Recombinant hirudin and α_2-AP were purchased from Sigma-Aldrich Company Ltd. (Poole, UK) Murine antihuman TF mAb 5G9 and 10H10 were provided by Dr. W. Ruf (Scripps Research Institute, La Jolla, CA; Ref. 14). Mouse antihuman uPAR mAb was provided by Dr. F. Lupu and Dr. V. Ellis (Thrombosis Research Institute, London).

Cell Lines. The 9 human pancreatic cell lines and 10 human breast cancer cell lines used in this study were obtained from Imperial Cancer Research Fund Cell Production Services (Clare Hall Laboratories, London. UK). MIAPaCa2, ASPC1, MCF7, T47D, and ZR75–1 were cultured in RPMI 1640 supplemented with 10% FCS and 20 mM L-glutamine in a humidified incubator with 5% CO_2 in air at 37°C. BxPC3, Capan2, T3M4, CfPac1, SW850, SW979, Panc1, BT-7, BT-20, MDA-MB231, MDA-MB453, and MDA-MB468 were cultured in E4 medium with 10% fetal bovine serum in a humidified incubator with 10% CO_2 in air at 37°C. MDA-MB415 and MDA-MB436 were cultured in E4 medium supplemented with 10% fetal bovine serum and 10 μg/ml insulin.

Received 4/27/98; accepted 7/30/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the Imperial Cancer Research Fund and Medical Research Fund and by a Medical Research Council Clinician Scientist Fellowship (to A. K. K.).

[2] To whom requests for reprints should be addressed, at Imperial Cancer Research Fund Molecular Oncology Unit, Imperial College School of Medicine, Hammersmith Campus, Du Cane Road, London W12 0NN, UK. Phone: 44-181-383-3257; Fax: 44-181-383-3258; E-mail: n.lemoinc@icrf.icnet.uk.

[3] The abbreviations used are: TF, tissue factor; FVIIa, factor VIIa; FVIIai, functionally inactivated FVIIa; uPA, urokinase; uPAR, uPA receptor; α_2-AP, α_2-antiplasmin; mAb, monoclonal antibody; FACS, fluorescence-activated cell-sorting.

Flow Cytometry. MIAPaCa2 and SW979 cells were dissociated into single-cell suspension in Ca^{2+}/Mg^{2+}-free PBS. Cells were then pelleted by centrifugation and washed with ice-cold PBS with 0.1% (w/v) BSA and 0.1% (w/v) sodium azide (FACS washing buffer) twice. Cells were pelleted again, resuspended in FACS washing buffer with the primary antihuman TF mAb (10H10) at 50 μg/ml for 30 min, washed twice, and then incubated with FITC-conjugated sheep antimouse IgG (Sigma-Aldrich Ltd., Poole, UK) for 30 min. Cells were then washed with FACS washing buffer three times and resuspended in 500 μl of FACS washing buffer. Cell-associated fluorescence was quantified by flow cytometry with the FACS Vantage flow cytometer (Becton Dickinson Labware, San Jose, CA)

Northern Blot Analysis. The cells were plated at a density of 10^6 cells/100-mm dish and grown to 70–80% confluence in medium supplemented with 10% FCS. Before being used for stimulation experiments, cells were cultured in medium supplemented with 0.5% FCS for 48 h. Total cellular RNA samples were isolated from cells using QIAGEN RNeasy kit (QIAGEN Ltd., UK) according to the manufacturer's protocol. For Northern transfer, 10 μg of each RNA sample was denatured, subjected to electrophoresis in 1.2% formaldehyde-agarose gel, and transferred to a Hybond-N+ nylon membrane (Amersham Life Sciences, Amersham, UK) by capillary transfer. After transfer, RNA was cross-linked onto the membrane by UV irradiation. Hybridization was performed in Rapid-hyb buffer (Amersham RPN 1636) and heat-denatured herring sperm DNA at 64°C for 2 h. The membranes were washed twice with 2 × SSC-0.1% SDS at 64°C, then once with 1 × SSC-0.1% SDS at 64°C. The membranes were autoradiographed against Amersham Hyperfilm at −80°C for 2 to 5 days. The human cDNA probes of uPA (600-bp fragment cut by *EcoRI/Hin*dIII), uPAR (1113-bp fragment cut by *XbaI/Eco*RI), and PAI-1 (504-bp fragment cut by *Pst*I), which were subcloned into pBluescript M13+, were kindly provided by Dr. M. F. Scully (Thrombosis Research Institute, London). Probes were radiolabeled with [α-^{32}P]dCTP (Amersham) using a DNA Labeling Kit (-dCTP; Pharmacia Biotech). Relative signal intensity was determined on a Molecular Imager (Bio-Rad Laboratories, CA94547) using Molecular Analyst Software Version 2.1.

Western Blot Analysis. Cells were washed with ice-cold Ca^{2+}/Mg^{2+}-free PBS, then treated with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.02% sodium azide, 1% Triton X-100, 1 μg/ml aprotinin, and freshly added 100 μg/ml phenylmethylsulfonyl fluoride). The lysate was centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was collected and electrophoresed on a 10% SDS-polyacrylaminde gel, followed by transfer of the proteins onto Hybond™ ECL™ Nitrocellulose membrane (Amersham) using a semi-dry transfer system. The blots were incubated with antihuman uPAR mAB, and bands were detected using the ECL Western blotting system (Amersham) according to the manufacturer's protocol.

Matrigel Migration Assay. SW979 migration was studied using the Transwell Cell Culture Chamber (12-mm diameter, 12.0-μm pore size) purchased from Costar (Cambridge, MA). The chambers were coated with Matrigel (Becton Dickinson Labware, Oxford, UK) that was diluted with 2 volumes of OPTI-MEM (Life Technologies Ltd., Paisley, UK). Two × 10^5 cells were seeded with 500 μl of medium supplemented with 0.5% FCS and incubated in 10% CO_2 at 37°C for 72 h; then the matrigel on the upper surface of the filters was removed. The filters were fixed with 100% methanol and stained with H&E. The number of cells that had migrated to the lower surface was counted in six random fields using a light microscope (×400).

Statistical Analysis. Statistical analysis of matrigel migration assay was performed by using ANOVA and unpaired Student's *t* test. The Kruskal-Wallis test was done for determination of correlation of gene expression. All statistics were performed in two-sided tests. $P < 0.05$ was considered significant.

RESULTS

In Northern blot analysis, TF mRNA, especially the 2.2- and 3.1-kb species, was highly expressed in SW979, T3M4, ASPC1, and CfPac1 cell lines, moderately expressed in BxPC3 and SW850 cell lines, but not expressed in MIAPaCa2, Panc1, and CaPan2 cell lines (Fig. 1*A*). In FACS analysis, TF antigen was highly expressed in the SW979 cell line but not expressed in the MIAPaCa2 cell line (Fig. 1*B*). This result was consistent with the results of Northern blot analysis.

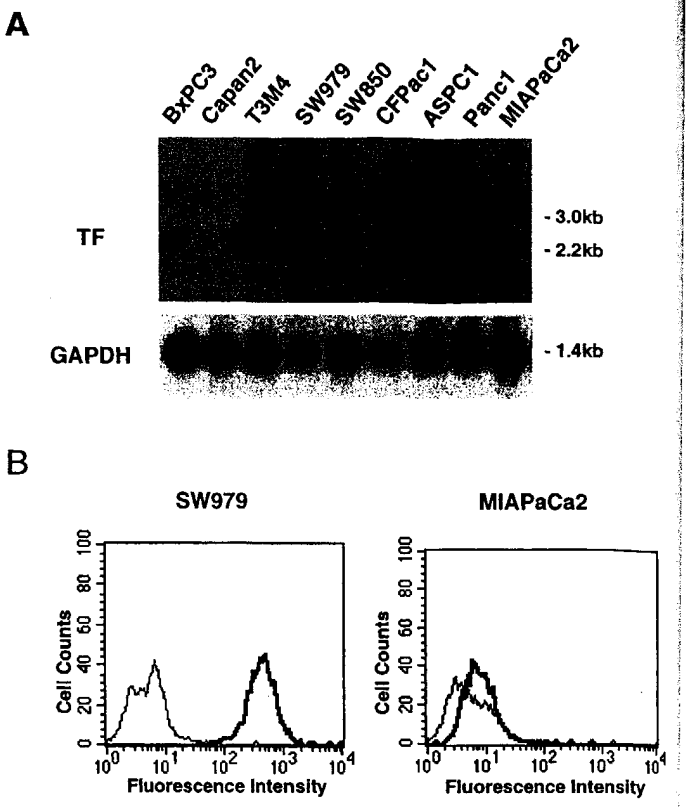


Fig. 1. TF expression in pancreatic cancer cell lines in Northern blot and FACS analysis. *A*, TF mRNA expression in Northern blot analysis. *B*, FACS analysis of TF expression in SW979 cells and MIAPaCa2 cells, respectively.

Cell-specific expression of uPAR, uPA, and *PAI-1* gene induced by FVIIa was examined by Northern blot analysis. In SW979 cells that highly express TF cell surface receptor, uPAR mRNA was increased after treatment with FVIIa in a dose-dependent manner, whereas uPA and PAI-1 mRNA were not increased (Fig. 2*A*). In the analysis of relative signal intensity using a Molecular Imager, expression of *uPAR* gene was approximately 2.7-fold increased by treatment with 100 nM FVIIa compared to control. The affinity of TF for FVIIa is known to be in the nanomolar range from previous studies (13). This up-regulation of uPAR mRNA was not found after treatment with FVIIa*i*, an antagonist of FVIIa under the same conditions (Fig. 2*A*). In the MIAPaCa2 cell line, which does not express detectable levels of TF, up-regulation of uPAR mRNA was not observed after treatment with FVIIa (Fig. 2*B*). In time course studies, uPAR expression was maximally increased to 5-fold over baseline 2–4 h after treatment with 100 nM FVIIa and decreased to the endogenous level by 48 h after treatment with FVIIa in SW979 cells (Fig. 3*A*). In contrast, levels of uPA, PAI-1 3.4-kb and PAI-1 2.2-kb transcription were increased less than 1.3-fold 2–4 h after treatment. We also performed Western blotting with uPAR mAb, which confirmed that the expression of protein (M_r 60,000) was increased compared to control after incubation with FVIIa for 3–24 h (Fig. 3*B*).

Incubation of SW979 cells with the neutralizing anti-TF mAb 5G9 resulted in complete inhibition of *uPAR* gene expression induced by FVIIa, whereas the nonneutralizing anti-TF mAb, 10H10, had no effect on *uPAR* gene up-regulation (Fig. 4). These results suggest that the binding of FVIIa to TF on cell surface is necessary to induce the gene up-regulation.

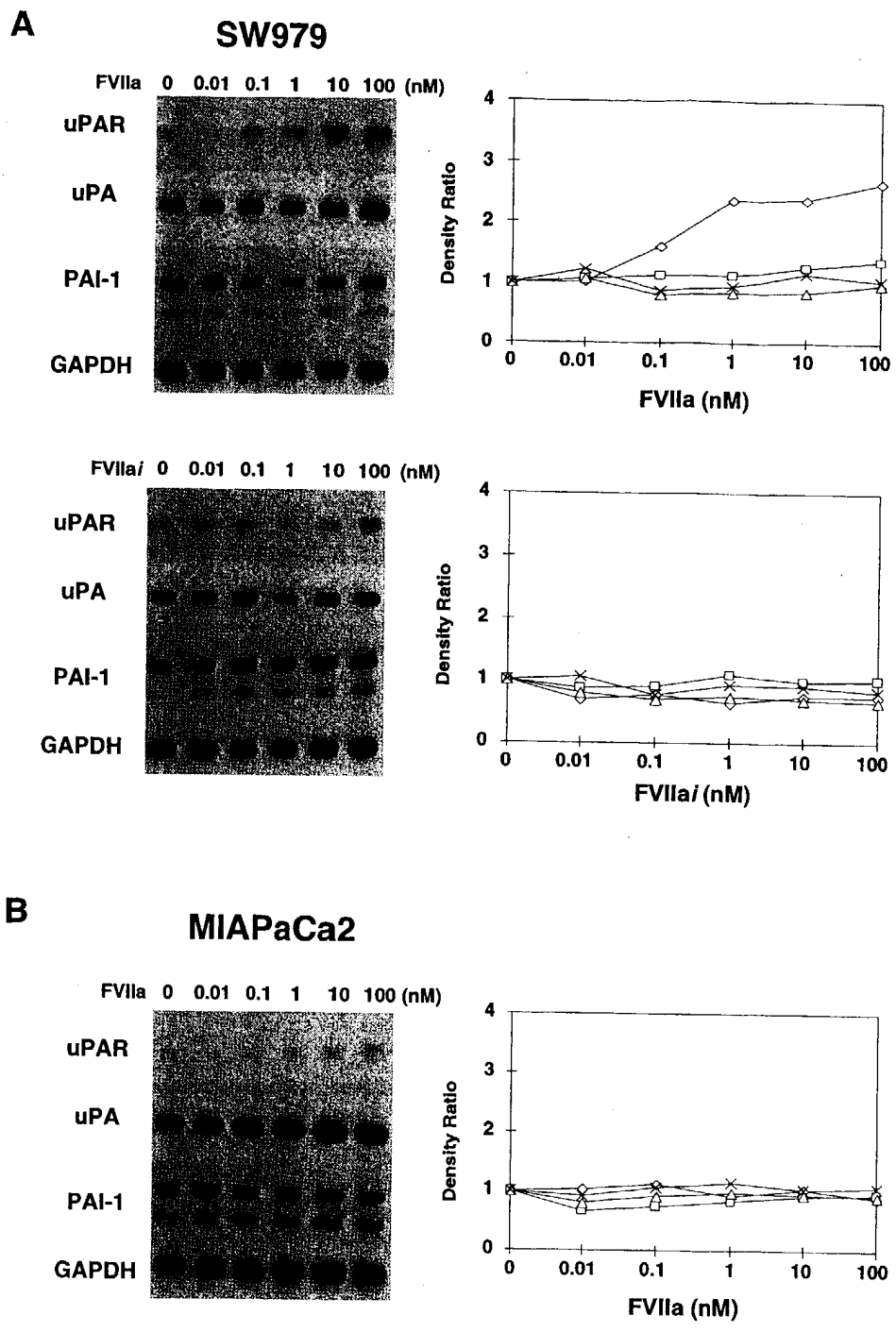


Fig. 2. Northern blot analysis of uPAR, uPA, and PAI-1 in SW979 cell line (*A*) and MIAPaCa2 cell line (*B*). Five × 10^5 cells were plated in 10-cm dishes and cultured for 24 h, then washed with PBS three times. After adding medium supplemented with 0.5% FCS, cells were cultured for 48 h and washed with medium without FCS three times before adding 10 ml of medium supplemented with 0.5% FCS and various concentrations of FVIIa or FVIIa*i* for 6 h. Relative density of radioactive signal was determined on a Molecular Imager (Bio-Rad Laboratories, CA94547) using Molecular Analyst Software Version 2.1. and standardized by using the relative density of GAPDH signal. ◇, uPAR; □, uPA; △, PAI-1 3.4 kb; and ×, PAI-1 2.2 kb.

To determine whether this up-regulation of uPAR mRNA was induced by factor X activation or thrombin generation triggered by the FVIIa-TF complex, the cells were treated with FXa. Treatment of SW979 cells with FXa did not up-regulate the expression of uPAR gene, while treatment with FVII induced the same level of uPAR gene expression as that with FVIIa (Fig. 5). The expression of uPAR induced by treatment with FVIIa was not affected by preincubation with the thrombin inhibitor, hirudin (Fig. 6). Treatment with α-thrombin or hirudin itself caused slightly (approximately 1.3-fold) increased expression of uPAR mRNA compared to control in SW979 cells. This effect was not significant compared to FVIIa treatment. These data indicate that neither factor X activation nor thrombin generation are implicated in the specific up-regulation of uPAR gene on SW979 cells, but the active-site of FVIIa might be important for up-regulation of the uPAR gene through the TF-FVIIa pathway.

To examine whether the specific up-regulation of uPAR induced by the treatment with FVIIa is associated with an invasive phenotype, the matrigel migration assay was performed. The migration of SW979 cells was increased by treatment with FVIIa in a dose-dependent manner, but not increased by FVIIa*i* (Fig. 7*A*). This increased migration was completely inhibited not only by the neutralizing anti-TF mAb, 5G9, but also by the specific plasmin inhibitor, α_2-AP (Fig. 7*B*). However, hirudin did not inhibit the increased migration mediated by FVIIa-TF complex. Moreover, hirudin itself enhanced the cell migration to some degree in our

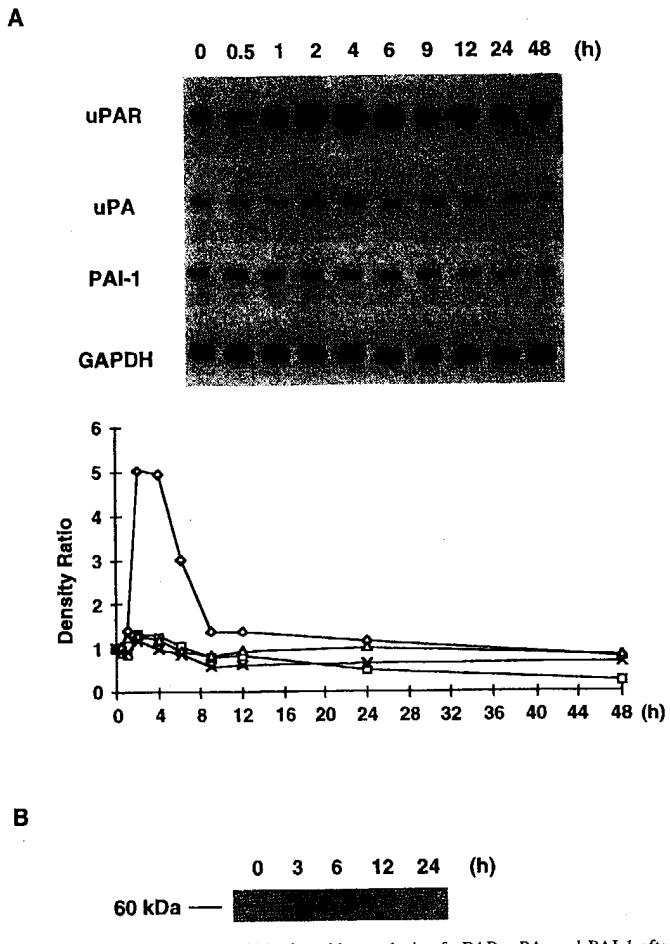


Fig. 3. Time course study of Northern blot analysis of uPAR, uPA, and PAI-1 after treatment with FVIIa (*A*). Five × 10^5 cells were plated in 10-cm dishes and cultured for 24 h, then washed with PBS three times. After adding medium supplemented with 0.5% FCS, cells were cultured for 48 h and washed with medium without FCS three times before adding 10 ml of medium supplemented with 0.5% FCS and 100 nM FVIIa. ◇, uPAR; □, uPA; △, PAI-1 3.4 kb; and ×, PAI-1 2.2 kb. Western blot analysis of uPAR expression after treatment with FVIIa under the same conditions (*B*). Cells were treated with 100 nM FVIIa. Cell lysates (30 μg protein) were analyzed by Western blotting using antihuman uPAR mAb.

experiments. These results suggest that the specific up-regulation of uPAR induced by the treatment with FVIIa could increase SW979 cell migration *in vitro*.

To compare endogenous expression levels of *TF* gene and uPAR, uPA and PAI-1, total RNA was extracted from 9 pancreatic cancer cell lines and 10 breast cancer cell lines that were cultured in medium with 10% FCS. We found that there was significant correlation between the expression levels of TF mRNA and uPAR mRNA ($P < 0.05$) in these 19 human cancer cell lines, whereas the expression of neither uPA mRNA nor PAI-1 mRNA was correlated with the expression of TF mRNA (Table 1).

DISCUSSION

Several recent studies have suggested the involvement of TF overexpression in the metastatic tumor phenotype (1–3), but thus far there have not been any reports that could explain how TF could influence invasive properties. There are two broad possibilities to explain this issue: (*a*) TF-FVIIa complex-driven initiation of the coagulation might activate a downstream-proteinase activated receptor or the thrombin receptor. Fisher *et al.* (15) showed that TF-initiated thrombin generation activated signaling through the thrombin receptor on malignant melanoma cells, although there is another report that suggested that the metastatic effect induced by TF expression did not involve products of the coagulation cascade (2); or (*b*) binding of FVIIa to TF may directly induce signals that are relevant to the metastatic tumor phenotype. Recently, Pendurthi *et al.* (16) used the differential display technique to show up-regulated transcription of poly(A)polymerase gene induced in human fibroblast cells by incubation with FVIIa. Poulsen *et al.* (17) demonstrated mitogen-activated protein kinase (MAPK) activation on exposure of baby hamster kidney cells to FVIIa that could not be blocked by tick anticoagulant protein and was not seen on exposure to FXa. This evidence supports the possibility that TF-mediated signal transduction events may involve up-regulation of cancer-related genes.

During cell migration, expression of the plasminogen activator system is up-regulated in several types of cells. uPAR is attached to the cell membrane by a glycosyl phosphatidyl inositol anchor that is added during posttranslational processing and that also involves COOH-terminal truncation of the primary translation product (18, 19). Binding of uPA to uPAR accelerates uPA activation from an enzymatically inactive proenzyme (pro-uPA). uPAR is also reported to be a genuine receptor that induces intracellular signal transduction events that lead to tumor cell activation. Soluble uPAR variants, which do not have the GPI anchor, have been reported (20, 21). uPA and uPAR are usually found at the leading cell edge at which plasminogen activity is accumulated to facilitate migration (22, 23). Interactions of PAI-1 and uPAR with the extracellular matrix protein vitronectin (VN) and integrin receptors have been reported (24–26). Overexpression

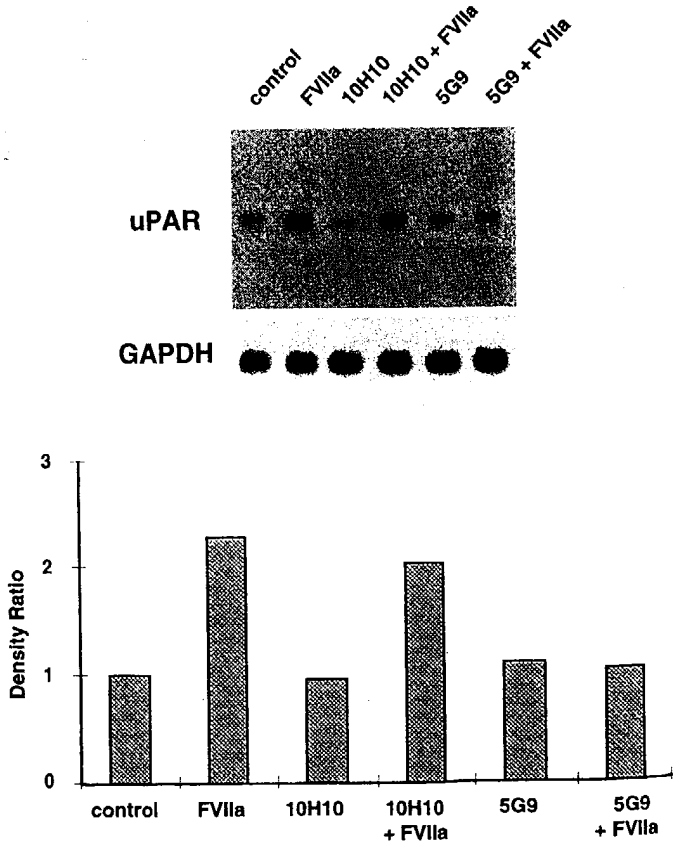


Fig. 4. Inhibition of *uPAR* gene expression using anti-TF mAbs. Cells were incubated with or without FVIIa 100 nM in the condition medium supplemented with 0.5% FCS for 6 h. The neutralizing anti-TF antibody, 5G9 (20 μg/ml), and nonneutralizing anti-TF antibody, 10H10 (20 μg/ml), were coincubated with FVIIa.

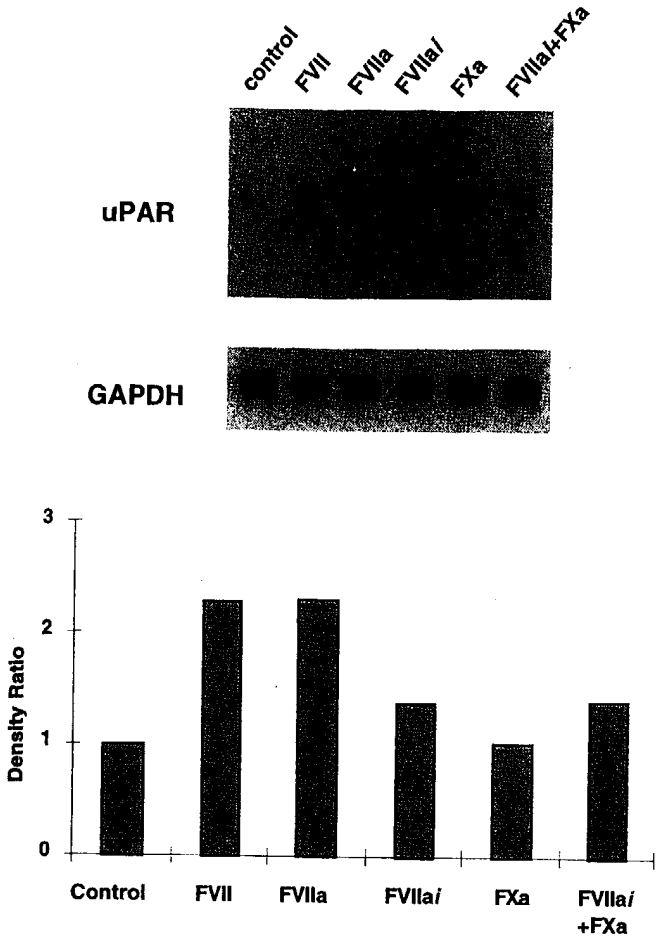


Fig. 5. Effects of FVII, FVIIa, FVIIai, and FXa in *uPAR* gene expression. SW979 cells were incubated with FVII 100 nM, FVIIa 100 nM, FVIIai 100 nM, FXa 100 nM, and FVIIai 100 nM and FXa 100 nM in the condition medium supplemented with 0.5% FCS for 6 h.

of uPAR has been reported in several types of human carcinoma including pancreatic carcinoma (27–30). Recently, differential regulation of uPA, uPAR, and PAI-1 at both transcription and post-transcription levels was described in human cancer cell lines (10–12, 31).

In this study, we show that the binding of FVIIa to TF cell surface receptor specifically up-regulated *uPAR* gene expression in SW979 human pancreatic cells that express TF mRNA and TF protein at high levels. Up-regulation of *uPAR* gene expression was not found in MIAPaCa2 cells, which do not express detectable levels of TF. This response induced by FVIIa was blocked by the neutralizing anti-TF mAb, 5G9. These results indicate that the TF-FVIIa complex directly or indirectly induced intracellular signaling resulting in expression of uPAR, a factor involved in tumor invasion and metastasis. To examine whether activation of FXa leading to thrombin generation is involved in the up-regulation of *uPAR* gene, SW979 cells were incubated with FXa alone or with FXa and FVIIai, neither of which induced uPAR up-regulation. Furthermore, α-thrombin itself had no significant effect on *uPAR* gene expression. The failure of FVIIai to up-regulate *uPAR* gene expression despite its 10-fold increased affinity for binding to TF is an interesting observation. It is possible that the active-site of FVIIa may play an important role in induction of the intracellular signaling through the TF-FVIIa complex or the activation of another downstream proteinase-activated receptor.

Several reports have shown that up-regulation of uPAR enhances migration not only on tumor cells (8, 9) but also on endothelial cells (32). In the Matrigel cell migration assay, the migration of SW979 cells was increased by incubation with FVIIa in a dose-dependent manner, and FVIIa-induced cell migration was completely inhibited by the neutralizing anti-TF mAb 5G9. To confirm whether the FVIIa-induced cell migration is caused by the enhanced expression of uPAR that binds endogenous uPA on the cell surface and enhances plasmin generation to mediate degradation of the Matrigel, cells were incubated with FVIIa in the presence of the specific plasmin inhibitor α_2-AP. FVIIa-induced cell migration was reduced to basal levels by coincubation with α_2-AP. In contrast, cell migration was increased by coincubation with FVIIa in the presense of hirudin. These results are consistent with the evidence of enhanced uPAR expression induced by the TF-FVIIa pathway leading to activation of the plasminogen activator system to facilitate cell invasion.

We also demonstrated that endogenous expression levels of TF were significantly correlated with those of uPAR in 19 human cancer cell lines ($P < 0.05$). This result suggests that not only binding of FVIIa to TF but also expression of TF itself may be important for the regulation of *uPAR* gene expression. In recent studies, Carmeliet *et al.* (33) demonstrated that inactivation of the TF gene resulted in the failure of blood vessel formation leading to embryonic death in knockout mice. Rosen *et al.* (34) showed that mice lacking FVIIa developed normally but suffered fatal perinatal bleeding. Hence, expression of TF appears to be crucial for vessel development, and FVIIa plays an important role in sustaining TF function for mainte-

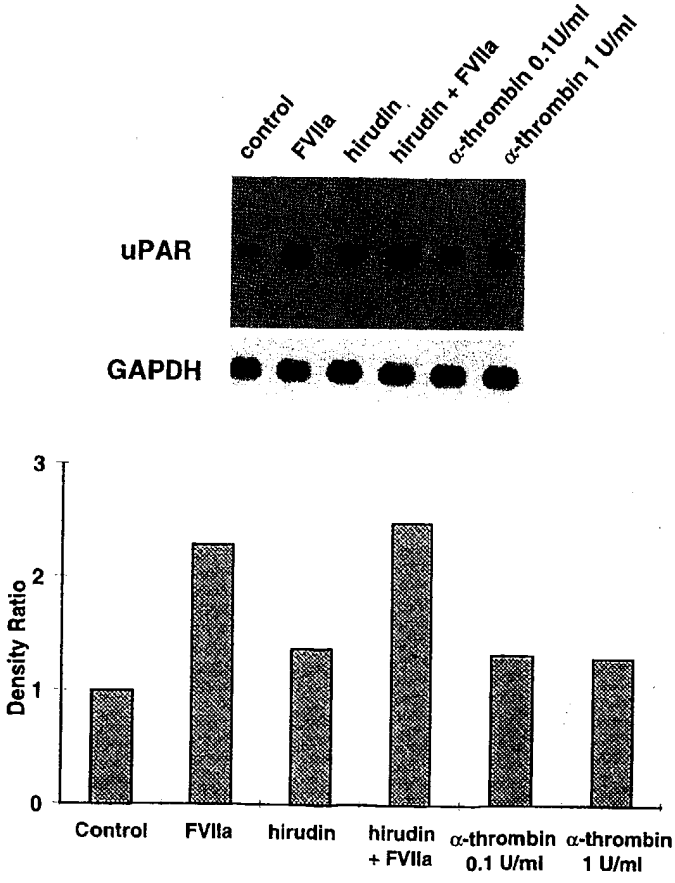


Fig. 6. Effects of hirudin and α-thrombin in *uPAR* gene expression. SW979 cells were incubated with hirudin (1 unit/ml) in a presence or absence of FVIIa (100 nM) in the condition medium supplemented with 0.5% FCS for 6 h. Cells were also incubated with α-thrombin (0.1 or 1 unit/ml) in the condition medium supplemented with 0.5% FCS for 6 h.

nance of vessels. In terms of the contributions of TF expression and FVIIa binding in influencing gene expression, our new evidence may be consistent with these observations.

Recently several reports demonstrated an association between TF expression and tumor grade. Kakkar *et al.* (35) showed that TF is not expressed in normal pancreatic tissue, whereas in pancreatic carcinoma, expression increases with progressive tumor dedifferentiation. In this study, 6 of 9 pancreatic cancer cell lines and 5 of 10 breast cancer cell lines expressed significant levels of TF mRNA. Clinically, pancreatic cancer is associated with a high frequency of thrombotic complications that may result from activation of the extrinsic pathway of blood coagulation and higher circulating levels of FVIIa (36). From this aspect, TF could be one of the most attractive targets for cancer therapy using not only anti-TF antibody to induce tumor vascular thrombosis (37) but also gene therapy aimed at down-regulation of *TF* gene to prevent tumor metastasis.

In conclusion, we have demonstrated that the binding of FVIIa to TF up-regulates uPAR expression and results in increased tumor migration by pancreatic cancer cells. Moreover, increased TF expression on the cell surface is associated with elevated expression of *uPAR* gene in human cancer cell lines. This is the first report to propose a potential molecular mechanism of TF-FVIIa complex-mediated signal transduction that can explain TF-induced tumor invasion and metastasis.

Table 1 *Result of Northern blot in pancreatic cancer cells and breast cancer cells*

	TF	uPAR[a]	PAI-1	uPA
Pancreas Cancer Cells				
SW979	+++[b]	++	++	++
ASPC1	++	++	±	++
T3M4	++	+	±	±
Cf Pac1	++	+	±	+++
SW850	+	+	±	+
BxPC3	+	+	+	++
MIAPaCa2	±	++	+	++
Panc1	±	±	++	++
CaPan2	±	±	+++	++
Breast Cancer Cells				
MDA-MB231	+++	++	++	++
MDA-MB436	+	++	+++	++
MDA-MB415	+	+	+	+
BT-20	+	+	±	±
MDA-MB468	+	+	±	±
BT475	±	±	±	±
MDA-MB453	±	±	±	±
MCF7	±	±	±	±
T47D	±	±	±	±
ZR75-1	±	±	±	±

[a] Statistically significant correlation between TF mRNA expression and uPAR mRNA expression, $P < 0.05$ (Kruskal-Wallis test).

[b] ±, almost undetectable expression; +, low level expression, ++, moderate expression; +++, high expression.

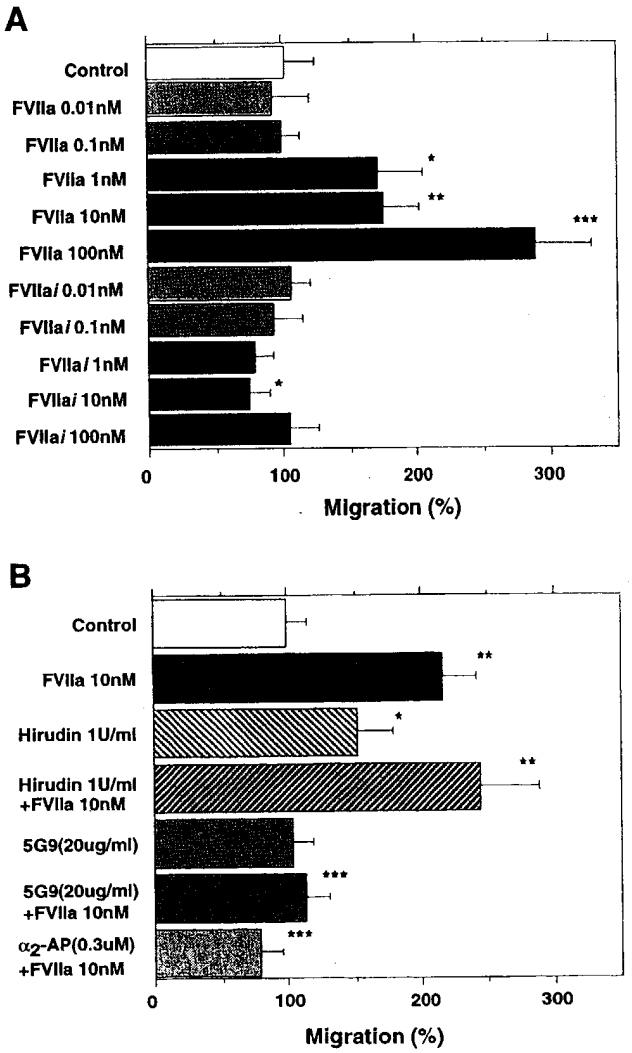


Fig. 7. Matrigel migration assay in SW979 cell lines. Two × 10^5 cells were seeded in upper chambers and cultured in 0.5% FCS for 72 h. The number of cells that migrated to lower surface was counted in six fields under the light microscope (×400). (*A*) *, $P < 0.01$ *versus* control; **, $P < 0.001$ *versus* control; and ***, $P < 0.0001$ *versus* control. (*B*) *, $P < 0.001$ *versus* control; **, $P < 0.0001$ *versus* control; and ***, $P < 0.0001$ *versus* FVIIa 10 nM. All experiments were performed three times, and typical data are shown.

ACKNOWLEDGMENTS

We thank Dr. W. Ruf (Scripps Research Institute, La Jolla, CA) for antihuman TF mAb 5G9 and 10H10 and Drs. F. Lupu and V. Ellis (Thrombosis Research Institute, London) for antihuman uPAR mAb.

REFERENCES

1. Mueller, B. M., Reisfeld, R. A., Edgington, T. S., and Ruf, W. Expression of tissue factor by melanoma cells promotes efficient hematogenous metastasis. Proc. Natl. Acad. Sci. USA, *89:* 11832–11836, 1992.
2. Bromberg, M. E., Konigsberg, W. H., Madison, J. F., Pawashe, A., and Garen, A. Tissue factor promotes melanoma metastasis by a pathway independent of blood coagulation. Proc. Natl. Acad. Sci. USA, *92:* 8205–8209, 1995.
3. Kataoka, H., Uchino, H., Asada, Y., Hatakeyama, K., Nabeshima, K., Sumiyoshi, A., and Koono, M. Analysis of tissue factor pathway inhibitor expression in human colorectal carcinoma cell lines and metastatic sublines to the liver. Int. J. Cancer, *72:* 878–884, 1997.
4. Bazan, J. F. Structural design and molecular evolution of a cytokine receptor superfamily. Proc. Natl. Acad. Sci. USA, *87:* 6934–6938, 1990.
5. Ruf, W., Schullek, J. R., Stone, M. J., and Edgington, T. S. Mutational mapping of functional residues in tissue factor: identification of factor VII recognition determinants in both structural modules of the predicted cytokine receptor homology domain. Biochemistry, *33:* 1565–1572, 1994.
6. Zioncheck, T. F., Roy, S., and Vehar, G. A. The cytoplasmic domain of tissue factor is phosphorylated by a protein kinase C-dependent mechanism. J. Biol. Chem., *267:* 3561–3564, 1992.
7. Röttingen, J., Enden, T., Camerer, E., Iversen, J., and Prydz, H. Binding of human factor VIIa to tissue factor induces cytosolic Ca^{2+} signals in J82 cells, transfected COS-1 cells Madin-Darby Canine Kidney Cells and in human endothelial cells induced to synthesize tissue factor. J. Biol. Chem., *270:* 4650–4660, 1995.
8. Kariko, K., Kuo, A., Boyd, D., Okada, S. S., Cines, D. B., and Barnathan, E. S. Overexpression of urokinase receptor increases matrix invasion without altering cell migration in a human osteosarcoma cell line. Cancer Res., *53:* 3109–3117, 1993.
9. Hudson, M. A., and McReynolds, L. M. Urokinase and the urokinase receptor: association with in vitro invasiveness of human bladder cancer cell lines. J. Natl. Cancer Inst., *89:* 709–717, 1997.
10. Dang, J., Wang, Y., and Doe, W. F. Sodium butyrate inhibits expression of urokinase and its receptor mRNAs at both transcription and post-transcription levels in colon cancer cells. FEBS Lett., *359:* 147–150, 1995.
11. Lund, L. R. Expression of urokinase-type plasminogen activator, its receptor and type-1 plasminogen activator inhibitor is differently regulated by inhibitors of protein synthesis in human cancer cell lines. FEBS Lett., *383:* 139–144, 1996.
12. Antalis, T. M., and Reeder, J. A. Butyrate regulates gene expression of the plasminogen activating system in colon cancer cells. Int. J. Cancer, *62:* 619–626, 1995.

13. Sørensen, B. B., Persson, E., Freskgård, P-O., Kjaika, M., Ezban, M., Williams, T., and Rao, L. V. M. Incorporation of active site inhibitor in factor VIIa alters the affinity for tissue factor. J. Biol. Chem., *272:* 11863–11868, 1997.
14. Ruf, W., Rehemtulla, A., and Edgington, T. S. Antibody mapping of tissue factor implicates two different exon-encoded regions in function. Biochem. J., *278:* 729–733, 1991.
15. Fischer, E. G., Ruf, W., and Mueller, B. M. Tissue factor-initiated thrombin generation activates the signaling thrombin receptor on malignant melanoma cells. Cancer Res., *55:* 1629–1632, 1995.
16. Pendurthi, U. R., Alok, D., and Rao, L. V. M. Binding of factor VIIa to tissue factor induces alterations in gene expression in human fibroblast cells: up-regulation of poly(A) polymerase. Proc. Natl. Acad. Sci. USA, *94:* 12598–12603, 1997.
17. Poulsen, L. K., Jacvobsen, N., Sorenson, B. B., Bergenhem, N. C. H., Kelly, J. D., Foster, D. C., Thastrup, O., Ezban, M., Petersen, L. C. Signal transduction via the mitogen-activated protein kinase pathway induced by binding of coagulation factor VIIa to tissue factor. J. Biol. Chem., *273:* 6228–6232, 1998.
18. Ploug, M., Ronne, E. N., Jensen, A. L., Blasi, F., and Dano, K. Cellular receptor for urokinase plasminogen activator. Carboxyl-terminal processing and membrane anchoring by glycosyl-phosphatidylinositol. J. Biol. Chem., *266:* 1926–1933, 1991.
19. Moller, L. B., Ploug, M., and Blasi, F. Structural requirements for glycosyl-phosphatidylinositol-anchor attachment in the cellular receptor for urokinase plasminogen activator. Eur. J. Biochem., *208:* 493–500, 1992.
20. Lau, H. K., and Kim, M. Soluble urokinase receptor from fibrosarcoma HT-1080 cells. Blood Coagul. Fibrinolysis, *5:* 473–478, 1994.
21. Pedersen, N., Schmitt, M., Ronne, E., Nicoletti, M. I., Hoyer-Hansen G., Conese, M., Giavazzi, R., Dano, K., Kuhn, W., Janicke, F., and Blasi, F. A ligand-free, soluble urokinase receptor is present in the ascitic fluid from patients with ovarian cancer. J. Clin. Invest., *92:* 2160–2167, 1993.
22. Odekon, L. E., Blasi, F., and Rifkin, D. B. Requirement for receptor-bound urokinase in plasmin-dependent cellular conversion of latent TGF-β to TGF-β. J. Cell. Physiol., *158:* 398–407, 1994.
23. Okada, S., Tomaszewski, J. E., and Barnathan, E. S. Migrating vascular smooth muscle cells polarize cell surface urokinase receptors after injury in vitro. Exp. Cell Res., *217:* 180–187, 1995.
24. Nip, J., Rabbani, S. A., Shibata, H. R., and Brodt, P. Co-ordinated expression of the vitronectin receptor and the urokinase-type plasminogen activator receptor in metastatic melanoma cells. J. Clin. Invest., *95:* 2096–2103, 1995.
25. Xue, W., Mizukami, I., Todd, R. F., III, Petty, H. R. Urokinase-type plasminogen activator receptors associate with β-1 and β-3 integrins of fibrosarcoma cells: dependence on extracellular matrix components. Cancer Res., *57:* 1682–1689, 1997.
26. Carriero, M. V., Del Vecchio, S., Franco, P., Potena, M. I., Chiaradonna, F., Botti, G., Stoppelli, M. P., and Salvatore, M. Vitronectin binding to urokinase receptor in human breast cancer. Clin. Cancer Res., *3:* 1299–1308, 1997.
27. Cantero, D., Friess, H., Deflorin, J., Zimmermann, A., Brundler, M. A., Riesle, E., Korc, M., and Buchler, M. W. Enhanced expression of urokinase plasminogen activator and its receptor in pancreatic carcinoma. Br. J. Cancer, *75:* 388–395, 1997.
28. Duggan, C., Maguire, T., McDermott, E., O'Higgins, N., Fennelly, J. J., and Duffy, M. J. Urokinase plasminogen activator and urokinase plasminogen activator receptor in breast cancer. Int. J. Cancer, *61:* 597–600, 1995.
29. Pyke, C., Kristensen, P., Ralfkiaer, E., Grondahl-Hansen J., Eriksen, J., Blasi, F., and Dano, K. Urokinase-type plasminogen activator is expressed in stromal cells and its receptor in cancer cells at invasive foci in human colon adenocarcinomas. Am. J. Pathol., *138:* 1059–1067, 1991.
30. Schmalfeldt, B., Kuhn, W., Reuning, U., Pache, L., Dettmar, P., Schmitt, M., Janicke, F., Hofler, H., and Graeff, H. Primary tumor and metastasis in ovarian cancer differ in their content of urokinase-type plasminogen activator, its receptor, and inhibitors types 1 and 2. Cancer Res., *55:* 3958–3963, 1995.
31. Shetty, S., Kumar, A., and Idell, S. Posttranscriptional regulation of urokinase receptor mRNA: identification of a novel urokinase receptor mRNA binding protein in human mesothelioma cells. Mol. Cell. Biol., *17:* 1075–1083, 1997.
32. Pepper, M. S., Sappino, A-P., Stocklin, R., Montesano, R., Orci, L., and Vassalli, J.D. Upregulation of urokinase receptor expression on migrating endothelial cells. J. Cell Biol., *122:* 673–684, 1993.
33. Carmeliet, P., Mackman, N., Moons, L., Luther, T., Gressens, P., Vlaenderen, I. V., Demunck, H., Kasper, M., Breier, G., Evrard, P., Muller, M., Risau, W., Edgington, T., and Collen, D. Role of tissue factor in embryonic blood vessel development. Nature (Lond.), *383:* 73–75, 1996.
34. Rosen, E. D., Chan, J. C. Y., Idusogie, E., Clotman, F., Vlasuk, G., Luther, T., Jalbert, L. R., Albrecht, S., Zhong, L., Lissens, A., Schoonjans, L., Moons, L., Collen, D., Castellino, F. J., and Carmeliet, P. Mice lacking factor VII develop normally but suffer fatal perinatal bleeding. Nature (Lond.), *390:* 290–294, 1997.
35. Kakkar, A. K., Lemoine, N. R., Scully, M. F., Tebbutt, S., and Williamson, R. C. N. Tissue factor expression correlates with histological grade in human pancreatic cancer. Br. J. Surg., *82:* 1101–1104, 1995.
36. Kakkar, A. K., DeRuvo, N., Chinswangwatanakul, V., Tebbutt, S., and Williamson, R. C. N. Extrinsic-pathway activation in cancer with high factor VIIa and tissue factor. Lancet, *346:* 1004–1005, 1995.
37. Huang, X., Molema, G., King, S., Watkins, L., Edgington, T. S., and Thorpe, P. E. Tumor infarction in mice by antibody-directed targeting of tissue factor to tumor vasculature. Science (Washington DC), *275:* 547–550, 1997.

Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 12598–12603, November 1997
Medical Sciences

Binding of factor VIIa to tissue factor induces alterations in gene expression in human fibroblast cells: Up-regulation of poly(A) polymerase

USHA R. PENDURTHI, DEORAJ ALOK, AND L. VIJAYA MOHAN RAO*

Department of Biochemistry, University of Texas Health Center, Tyler, TX 75710

Edited by Philip W. Majerus, Washington University School of Medicine, St. Louis, MO, and approved September 15, 1997 (received for review June 3, 1997)

ABSTRACT **Tissue factor (TF) is the cellular receptor for an activated form of clotting factor VII (VIIa) and the binding of factor VII(a) to TF initiates the coagulation cascade. Sequence and structural patterns extracted from a global alignment of TF confers homology with interferon receptors of the cytokine receptor super family. Several recent studies suggested that TF could function as a genuine signal transducing receptor. However, it is unknown which biological function(s) of cells are altered upon the ligand, VIIa, binding to TF. In the present study, we examined the effect of VIIa binding to cell surface TF on cellular gene expression in fibroblasts. Differential mRNA display PCR technique was used to identify transcriptional changes in fibroblasts upon VIIa binding to TF. The display showed that VIIa binding to TF either up or down-regulated several mRNA species. The differential expression of one such transcript, VIIa-induced up-regulation, was confirmed by Northern blot analysis. Isolation of a full-length cDNA corresponding to the differentially expressed transcript revealed that VIIa-up-regulated gene was poly(A) polymerase. Northern blot analysis of various carcinomas and normal human tissues revealed an over expression of PAP in cancer tissues. Enhanced expression of PAP upon VIIa binding to tumor cell TF may potentially play an important role in tumor metastasis.**

Tissue factor (TF) is the cellular receptor for an activated form of clotting factor VII(a) and factor VIIa/TF complex is the physiological initiator of the coagulation cascade (1). Tissue factor is constitutively expressed in many cells including fibroblasts and pericytes in and surrounding blood vessel walls (2, 3). However, TF is not normally expressed in cells within vasculature, such as monocytes and endothelial cells. Regulation of TF gene by a large and diverse group of molecules (4) raises the possibility that TF may participate in biological processes other than hemostasis, particularly in angiogenesis and tumor metastasis. Recently, several studies (5–7) provided *in vivo* experimental evidences of a role for TF in tumor metastatic process. It has been suggested that the metastatic effect of TF could involve transduction of an angiogenic signal for vascularization of developing tumors (6).

Several recent findings suggest that TF could function as a true receptor. Specific serine residues in the cytoplasmic tail of TF have been shown to be phosphorylated in cells following stimulation with protein kinase C activator (8). Rottingen *et al.* (9) showed that the binding of VIIa to TF induced cytosolic Ca^{2+} signals in J82 cells and in other cell types. More recently, Masuda *et al.* (10) showed that several polypeptides were transiently phosphorylated on tyrosine residues when cultured human monocytes were incubated with VIIa. However, so far no reports are available on whether VIIa binding to TF alters the rates of synthesis and/or the steady state levels of any cellular mRNAs and of the corresponding proteins.

The present study is carried out to test the hypothesis that the occupancy of TF receptor by its ligand induces an intracellular signal(s) that will trigger a selective expression or suppression of cellular gene expression. We have used differential display PCR methodology to identify alterations in gene expression in fibroblasts upon VIIa binding to cell surface TF. The results indicate that the expression of several transcripts are altered by VIIa binding to cell surface TF and one such alteration is the induction of poly(A) polymerase (PAP) gene that plays an important role in processing mRNA. Our data also show that PAP gene expression is up-regulated in many human cancer tissues, particularly in colon carcinoma.

MATERIALS AND METHODS

Reagents. RNAmap kit for mRNA differential display PCR was purchased from GenHunter corporation (Brookline, MA). AmpliTaq polymerase was from Perkin–Elmer, [α-^{35}S]dATP and [α-^{32}P]dCTP (3,000 Ci/mmol; 1 Ci = 37 GBq) were from DuPont/NEN, nick translation kit was from Amersham, TRI reagent was from the Molecular Research Center (Cincinnati); T4 DNA ligase, DNase I, and restriction enzymes were from New England Biolabs or Promega. Human monocyte (phorbol 12-myristate 13-acetate-treated) cDNA library was obtained from CLONTECH.

Proteins. Recombinant human VIIa, a gift from Novo-Nordisk (Copenhagen), was reconstituted in sterile water at a concentration of 1 mg/ml. Factor Xa was prepared by incubating purified factor X with Russell's viper venom followed by benzamidine Sepharose chromatography (11). Monospecific, polyclonal anti-human TF antiserum was raised in a rabbit (3) and the IgG fraction was separated by precipitation at 40% ammonium sulfate saturation, followed by DEAE/Affi-Gel-Blue chromatography.

Cell Culture. A fibroblast cell line, WI-38, was grown in T-75 flasks as described earlier (12). When cells reached 80–90% confluency, the monolayers were washed once with buffer A (10 mM Hepes, pH 7.5, containing 150 mM NaCl/4 mM KCl/11 mM glucose) containing 5 mM EDTA and then twice with the buffer not containing EDTA before the monolayers were subjected to specific treatments.

Tissue Specimens. A variety of normal and cancer tissues were obtained from Nissi Varki (Cancer Center Research

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

This paper was submitted directly (Track II) to the *Proceedings* office.
Abbreviations: TF, tissue factor; VIIa, activated form of clotting factor VII; PAP, poly(A) polymerase.
Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF002990).
*To whom reprint requests should be addressed. e-mail: rao@uthct.edu.

Tissue Core, University of California, San Diego). These tissues, obtained from surgical specimens and in some cases from autopsies, were collected within an hour of removal. The tissue samples were flash frozen and stored at ultra low temperature (below −135°C). The existence of malignant cells in carcinoma tissues was confirmed by hematoxylin/eosin staining.

RNA Isolation and DNase I Treatment. Total RNA was isolated using TRI reagent according to the guidelines provided by the manufacturer. Total RNA, 25 μg in 100 μl of 10 mM Tris·HCl, 50 mM KCl, 1.5 mM $MgCl_2$, and 10 units of RNase inhibitor, was treated with 10 units of DNase I for 20 min at 37°C. Then, the RNA was extracted once with phenol/chloroform (1:1, vol/vol), precipitated with ethyl alcohol/0.3 M sodium acetate, and the pellet was resuspended in 20 μl of diethyl pyrocarbonate-treated water.

mRNA Differential Display. Differential display of mRNA was performed as described (13, 14) with slight modifications. Briefly, DNA free total RNA (0.2 μg in 20 μl) was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (100 units) for 1 h at 37°C in the presence of dNTPs (each at 20 μM) and one of the four $T_{12}MN$ oligonucleotides (1 μM) (M = G, C, or A; N = G, A, T, or C) that prime Poly(A) region of mRNAs. The cDNAs generated were then amplified by PCR using 0.1 volume of the above reverse transcribed mixture in the presence of [α-^{35}S]dATP. The reaction mixture (20 μl) contained 2 μM of dNTPs, 1 μM of the same arbitrary $T_{12}MN$ oligonucleotide that was used in the reverse transcription reaction, 0.2 μM of a specific arbitrary 10-mer and 10 units of AmpliTaq DNA polymerase. Light mineral oil was overlaid and PCR reactions were performed in a Perkin–Elmer thermal cycler. The reaction cycles were: 94°C for 30 sec, 40°C for 2 min, 72°C for 30 sec for 40 cycles; 72°C for 5 min and soak at 4°C. One μl of loading dye was added to 5 μl of PCR samples and heated to 80°C for 2 min prior to loading on 6% polyacrylamide sequencing gel. The gels were run at 90 watts with constant current for 2 h. The gels were dried without fixation and exposed directly to the Reflection NEF 495 autoradiography film overnight at room temperature.

Band Recovery and Reamplification. After orienting the dried gel with the autoradiograph, the differentially expressed bands were excised from the dried gel. The gel slice was soaked in sterile water for 10 min and then boiled for 15 min. After centrifugation, the supernatant was transferred to a new tube and the DNA was precipitated in the presence of glycogen and sodium acetate. The pellet was rinsed with 85% ice cold ethyl alcohol and resuspended in 10 μl of water. Reamplification of these DNAs was performed using one-third of the recovered sample by PCR with the appropriate 5′ primers and 3′ primers. The conditions for the PCR were the same as the above except that dNTPs concentrations were increased to 25 μM and the radioisotope tracer was omitted from the reaction mixture. The PCR products were run on a 1.8% agarose gel to determine the size of each fragment. The DNA bands were excised from the gel and the DNAs were eluted from the gel by a standard freeze-squeeze method.

Northern Blot Analysis, Cloning, and Sequencing. Reamplified cDNA probes were labeled with [α-^{32}P]dCTP by nick-translation. Northern blot analysis was carried out using standard procedures. The differentially expressed transcripts were subcloned into Bluescript plasmid at *Eco*RV site after the addition of T overhangs (15). Subcloned inserts were isolated, confirmed for differential hybridization on Northern blots, and then sequenced using Sequenase Rapid Well DNA sequencing kit (United States Biochemical).

Construction of Human Colon Carcinoma cDNA Library and Isolation of a Differentially Expressed cDNA. Total RNA was extracted from human colon carcinoma tissue using TRI reagent. Poly(A)-rich RNA was isolated from the total RNA using an oligo(dT) cellulose column. Size selected (0.8 to >4.0 kb) double-stranded DNA was cloned into Uni-ZAP XR vector (Stratagene). Clones (2×10^6) of the unamplified library were screened with a radiolabeled band 11 cDNA insert (see *Results* for details) employing standard procedures. Twelve positive clones were obtained in the initial screening. In further screenings, the positive clones were plaque purified. Bluescript phagemid DNA was recovered from the Uni-ZAP with ExAssist helper phage (Stratagene) and the inserts were released by *Eco*RI and *Xho*I digestion. The cDNA inserts of several positive clones were partially sequenced using T3/T7 primers to obtain 5′ and 3′ terminal nucleotide sequences.

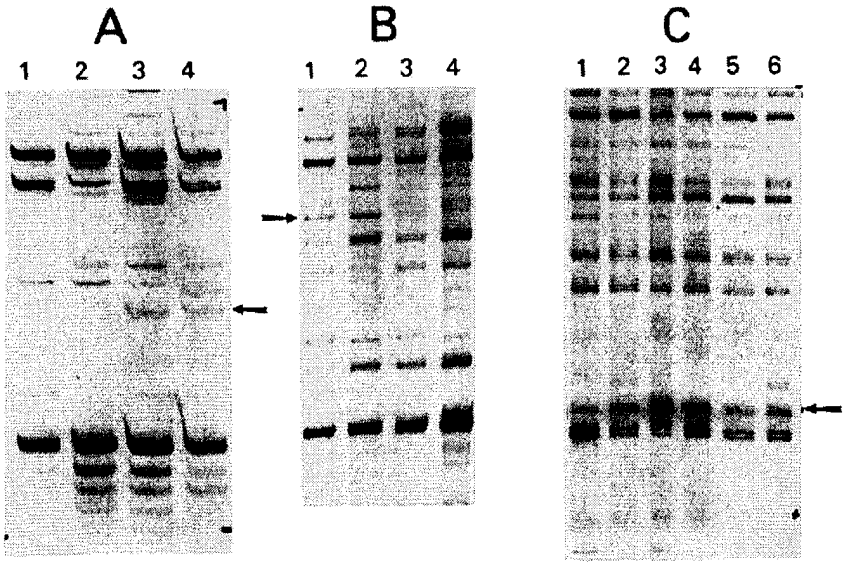


FIG. 1. Representative differential mRNA display PCR. Lanes 1 and 2, RNA from control cells; lanes 3 and 4, RNA from VIIa-treated cells; lane 5, RNA from Xa-treated cells; lane 6, RNA from anti-TF IgG-treated (200 μg/ml) cells prior to the addition of VIIa. The monolayers were treated for 90 min with a control buffer or the buffer containing VIIa or Xa, 10 nM. RNA samples were reverse transcribed and amplified with (*A*) $T_{12}MC$ and 5′-AGCTGACCGT-3′; (*B*) $T_{12}MA$ and 5′-GTGATCGGAC-3′; and (*C*) $T_{12}MT$ and 5′-AGCCAGCGAA-3′ primers. The differentially displayed bands are identified by arrows.

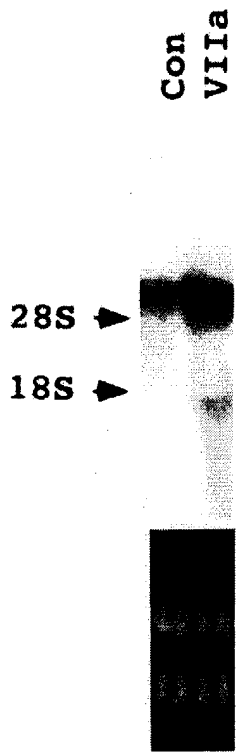


FIG. 2. Northern blot analysis confirming differential expression of a specific mRNA in VIIa-treated cells. WI-38 monolayers were treated with the control buffer or the buffer containing VIIa, 10 nM, for 90 min at 37°C. Total RNA was extracted from the cells and 15 μg of each RNA sample was used for Northern blot analysis. The blot was hybridized with a cDNA fragment of band 11 isolated from the display gel of Fig. 1*C*. (*Upper*) Northern blot analysis with band 11 cDNA. (*Lower*) Ethidium bromide staining of total RNA samples as controls for equal loading.

RESULTS

Differential mRNA Display. Fibroblast monolayers that constitutively express cell surface TF were cultured to 80–90% confluency in 75 mm^2 flasks and the monolayers were treated at 37°C with a control calcium containing buffer or the buffer containing VIIa, 10 nM. As additional controls, the monolayers were first incubated with anti-TF IgG, 200 μg/ml, for 20 min prior to the addition of VIIa or factor Xa was substituted for VIIa in the treatment. After a 90-min treatment period, total RNAs were isolated from the treated monolayers and the expression of mRNA was analyzed using combinations of 20 arbitrary 10-mers and four T_{12}MN primers. To minimize errors in the procedure and to avoid selection of artifact bands as differentially expressed transcripts, RNA samples prepared from two separate experiments were used as duplicates for each display. Each differential display lane yielded 35–60 discrete bands allowing evaluation of 2,800 to 4,800 mRNA species in total. Patterns of amplified cDNA species between the control and VIIa-treated samples were mostly identical, providing a reproducible uniform background over which specific differences could be observed (Fig. 1). In initial display, we identified 12 differentially expressed transcripts. To confirm the reproducibility of the differential expression, we repeated the display including reverse transcription/PCR amplification reactions using a new set of RNAs isolated from control and VIIa-treated cells on two different days. When the reactions were repeated for eight of the 12 selected bands, six of the eight bands showed reproducible differential expression. Two transcripts, designated as bands 11 and 16, were up-regulated, whereas four transcripts, designated as bands 12, 20, 27, and 28, were down-regulated upon VIIa binding to TF.

Northern Blot Analysis with Differentially Expressed Transcripts and Cloning of the Reamplified cDNA Fragments. The bands that showed VIIa-specific differential expression were excised from the gel, reamplified, labeled with [α-^{32}P]dATP, and used as probes in Northern blot analysis. The blots contained RNA isolated from the control and VIIa-treated WI-38 cells. Out of six probes tested, differential hybridization pattern was clearly evident with only one probe (band 11). With others, it was difficult to draw firm conclusions on their differential expression since the hybridization signals were either weak or hybridized to multiple mRNA species in both the control and VIIa-treated samples.

Next, the reamplified cDNA fragment of band 11 was cloned into the pBluescript SK+/− phagemid at *Eco*RV site after adding T overhang at 3′ end. Inserted cDNAs were isolated, radiolabeled, and used as a probe in Northern blot analysis to confirm the VIIa-specific up-regulation of the transcript. Several individual cDNA clones generated from band 11 showed an enhanced hybridization signal to a specific mRNA transcript of ≈4.5 kb in VIIa-treated cells (Fig. 2). In several experiments, we also observed a faint but specific hybridization to a mRNA transcript corresponding to 2.3 kb, in addition to the 4.5 kb, in VIIa-treated cells. Cloned cDNA fragments of band 11 that showed specific differential hybridization patterns on the Northern blots were sequenced. The nucleotide sequence showed that the band 11 transcript was flanked by mRNA mapping 10-mer primer sequences at the 5′ end and putative polyadenylation signals (T_{12}MN) at 3′ end (Fig. 3). Searching the GenBank and EMBL DNA databases using BLAST program revealed no homology sequences in the data bank.

Isolation of Full-Length cDNA for Band 11. A human monocyte, phorbol 13-myristate 12-acetate perturbed, λ gt11 cDNA library was screened with a cDNA probe generated from band 11. In an initial screening, one positive cDNA clone (≈0.7 kb) was obtained. The insert was isolated, radiolabeled, and used as a probe to confirm the differential expression in Northern blot analysis. The hybridization pattern of this probe was similar to that of hybridization pattern observed with the cDNA probe directly generated from the PCR product of band 11 (see Fig. 2). The 3′ sequence of this clone was identical to the sequence of band 11 cDNA fragment. However, the 0.7-kb cDNA insert also had no ORF. Rescreening of the monocyte library with the 0.7-kb insert did not yield any additional positive clones.

Next, we screened human colon carcinoma cDNA library to isolate a full-length cDNA corresponding to band 11 since a high level expression of this gene was found in human colon carcinoma tissue (see below). By screening 2 × 10^6 clones with

AGCCAGCGAA GAAAAAAGAT GGTCATACTA ACAGGTGAAA TGTACAAGGT GTCTGTGTGT
TTTGTGTAGC TTCAGAGTTA GATTGAAATT ACCAGGCACA GATTTAGTCT TGTCATTTTG
TTTACACATT GGGGAAAACA ATTCAGTTTA TTAAACGTTT CATGTAACTG CACCCAAGTT
TTGCCAAGCT GGAAACTTGG ACCTTTTCTG TGTAGTGACT TTTTAATTAT AGTTTTCATA
ACCTGGAGAT CAGACTGTTG CTTTCGCATG ATGTATGTAG TGTCTCATGA CTGGAGTTTG
CTTTGTTTTA TAGTATCTGT ACTCCTTGTA TTTTTCAAGA GCTATTTTGT AAACAGATGA
TGTATTTCTC CATTGAAAAC ACAATAAAAA AAAACAGCAC AAAAAAAAAA AA

FIG. 3. Nucleotide sequence of band 11 transcript. Flanking primer sequences are underlined.

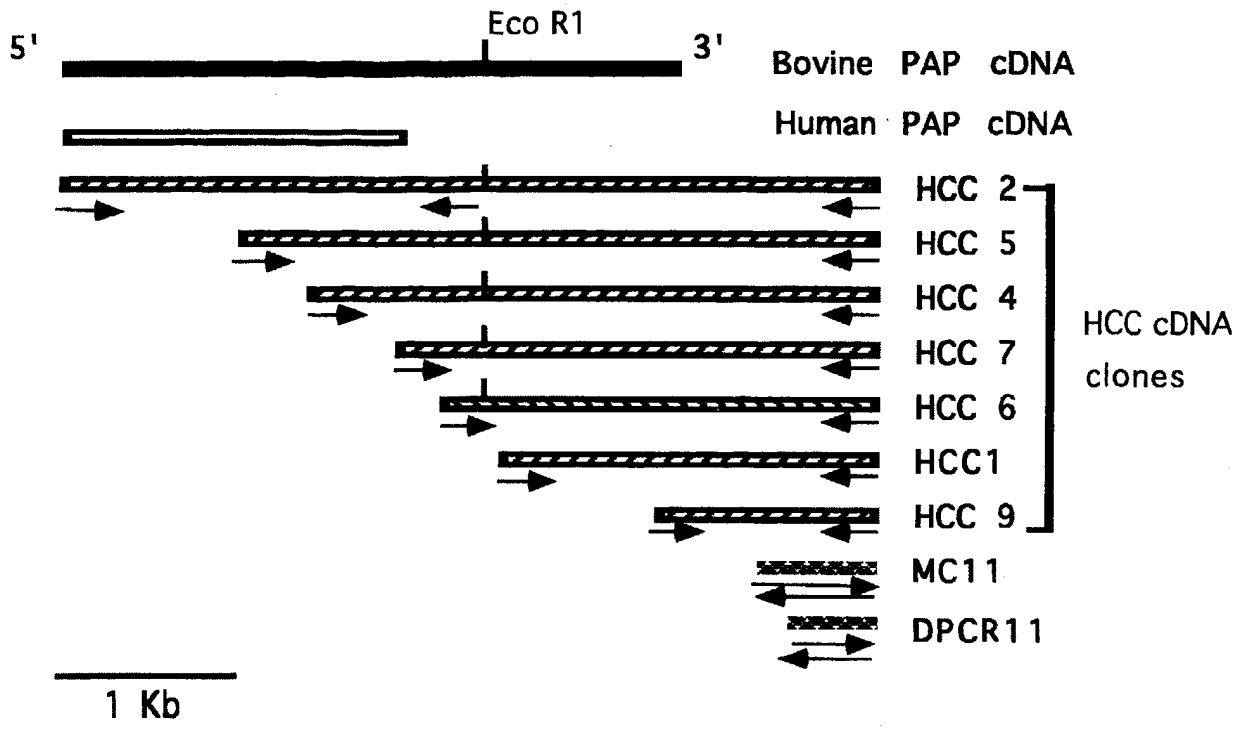


FIG. 4. The schematic representation of cDNA clones that were isolated from human colon carcinoma (HCC) cDNA library by using MC11 cDNA probe. The direction of arrows and the length of arrows under each clone represent the direction of the sequence and the length of the sequence obtained. Top bars represent the length of known cDNA sequences for the bovine PAP (solid) and the human PAP (open). DPCR 11 denotes a band 11 cDNA and MC11 denotes a cDNA clone corresponding to band 11 isolated from phorbol 13-myristate 12-acetate-treated human monocyte cDNA library.

the 700-bp insert isolated from phorbol 13-myristate 12-acetate-perturbed monocytic cDNA library yielded 12 positive clones. The estimated inserts size varied from 1.1–4.5 kb. Several of these inserts were sequenced partially to obtain 5′ and 3′ terminal nucleotide sequences. The limited sequences obtained showed a near 100% homology with human PAP (16) and ≈97% homology with bovine PAP (17). The longest PAP cDNA isolated was ≈4,500 bp, and was likely to be a full-length since it started with a 5′ untranslated region and ended with a Poly(A) tail at 3′. This cDNA clone was ≈2.5 kb and ≈1.1 kb longer than the previously isolated PAP cDNAs from human (16) and bovine (17) sources, respectively (Fig. 4).

To further strengthen our observations and to show that PAP was indeed differentially expressed in VIIa-treated cells, the full-length PAP cDNA insert was isolated, radiolabeled, and used to probe the RNA isolated from the control and VIIa-treated cells by Northern blot analysis. The data showed that PAP message was up-regulated in VIIa-treated cells (Fig. 5).

Increased Expression of Band 11 Transcript (PAP) in Cancer. RNA was isolated from a variety of normal and cancerous tissues and analyzed by Northern blot. The blot was probed with the 400-bp band 11 cDNA fragment that was originally isolated from the display. As seen in Fig. 6, the specific hybridization signal for a 4.5-kb mRNA transcript was much stronger for RNA isolated from cancer tissues than RNA isolated from their benign counterparts. The data also showed a faint hybridization of band 11 transcript to a 2.3-kb mRNA transcript in cancer tissues but not in normal tissues. The expression of band 11 transcript was particularly striking in colon carcinoma compared with a low level expression found in benign colon tissue. The increased expression of the band 11 transcript was also evident in many other malignant tissues, such as breast, ovary, and pancreas.

DISCUSSION

In many receptor-mediated processes, occupancy of the receptor by its ligand alters the rate of synthesis and steady state levels of many cellular RNAs and the corresponding proteins. The present study is the first to provide evidence for the binding of VIIa to TF receptor leads to specific alterations in cellular gene expression in cultured fibroblasts. In this study, we employed differential mRNA display method to identify VIIa-induced specific gene alterations. To avoid isolating mRNAs whose differential expression might have been simply related to artifacts in the lengthy procedure rather than specific for VIIa treatment, we routinely performed differential display PCR using two different RNA samples obtained on two different days. Further, in several display runs, we also included RNA samples obtained from the monolayers treated with factor Xa and the monolayers treated with anti-TF IgG before the monolayers were exposed to factor VIIa as additional controls. More importantly, we repeated differential display analysis two or three times for each potential "positives" and only those that were reproduced consistently were considered as potential transcripts whose expression was altered by factor VIIa binding to TF. Even with such stringent conditions, only one of the six selected differentially expressed transcripts showed VIIa-specific differential expression on Northern blot analysis. However, this finding was not entirely unexpected. Differential display signals not confirmed by Northern blot analysis were relatively common due to undetectable signals (18). It is possible that a PCR-amplified product from a display band can contain a number of distinct multiple cDNA fragments, similar in size, but derived from different genes. The problem can be confounded further if the transcript that shows differential hybridization is present in low abundance.

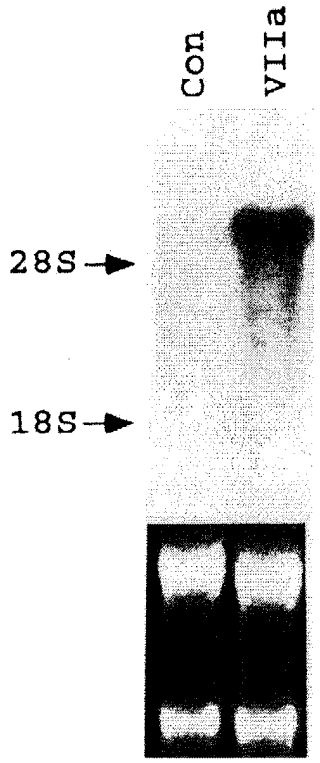


FIG. 5. Increased expression of PAP mRNA in fibroblasts treated with VIIa. WI-38 monolayers were treated with VIIa as described in Fig. 2. Total RNA was extracted from the cells and 15 μg of each RNA sample was used for Northern blot analysis. The blot was hybridized with a full-length PAP cDNA insert. Lane 1, RNA from control cells; lane 2, RNA from VIIa-treated cells. Bottom panel shows ethidium bromide staining of total RNA samples as controls for equal loading.

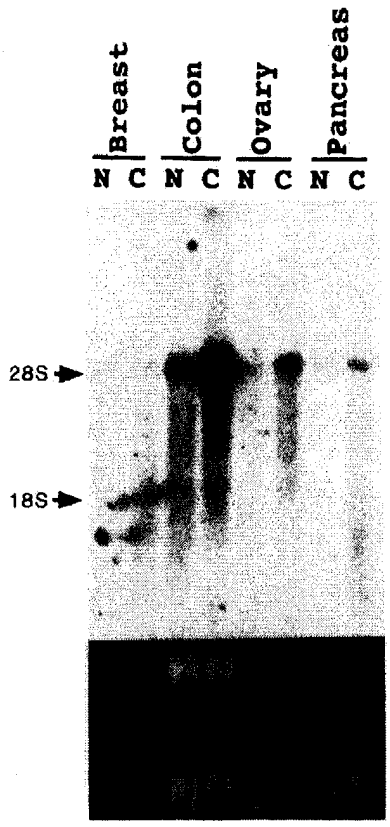


FIG. 6. Northern blot analysis of various cancer and benign tissues for the expression of band 11 transcript. Ten micrograms of total RNA from each tissue was loaded and the blot was hybridized with the 400-bp band 11 cDNA fragment. (*Bottom*) Ethidium bromide staining of total RNA samples as controls for equal loading. N, normal tissue; C, cancer tissue.

A specific transcript that was shown to be up-regulated in VIIa-treated cells (band 11) hybridized to two mRNA species of 4.5- and 2.3-kb on Northern blot analysis. Although the band 11 transcript hybridizes to the 4.5-kb transcript in RNA isolated from the control cells (not treated with VIIa), the intensity of the hybridization signal is lower in the controls compared with the VIIa-treated samples. The sequence of band 11 transcript did not match to any known sequences in the GenBank and EMBL databases. However, since the differential display method provides clones that contain only extreme 3′ regions of mRNA, the lack of homology with known sequences does not eliminate the possibility that the band 11 transcript may be homologous to known cDNAs whose 3′-untranslated region sequences were unavailable. Indeed, isolation a full-length cDNA clone corresponding to the band 11 transcript from human colon carcinoma cDNA library revealed that the VIIa-specific transcript we isolated from the differential display is the product of an already known gene, PAP.

PAP, both the mRNA and the protein, in mammalian cells exists in multiple forms (16). Northern blot analysis with RNA from HeLa cells and calf thymus showed three bands, 4.5, 2.4, and 1.3 kb (19). This could explain why our band 11 cDNA hybridized to two different mRNA species of 4.5 and 2.3 kb.

PAP plays a critical role in the synthesis of messenger RNA in eukaryotic cells. The main function of PAP is the addition of adenylate residues onto mRNA 3′ end following cleavage (20, 21). The length of polyadenylation is directly correlated to mRNA stability (22). Poly(A) tail shortening and its ultimate removal leads to mRNA degradation. In short lived mRNA, such as c-fos, poly(A) tail shortening is exceptionally rapid (23). Most of the oncogene and cytokines mRNA are also short lived. It is possible that increased expression of PAP could increase the polyadenylation of these mRNAs thereby increase their stability that ultimately leads to increased translation products. Such a mechanism would potentiate the action of many short-lived cytokines synthesized by tumor cells. However, further experiments are needed to substantiate that the observed increase in PAP in the cancer tissue is due to increased expression of PAP by the cancer cells and not the surrounding stromal cells or endothelial cells.

Recent studies showed that TF plays a role in angiogenesis and tumor metastasis (24). However, it is controversial whether TF coagulant function is required for tumor cell dissemination. Bromberg *et al.* (6) reported that a nonmetastatic human cell line became metastatic upon transfection with retrovirus carrying human TF cDNA to overexpress TF. Since metastases also occurred in mice injected with the melanoma cells expressing the extracellular TF mutant that was defective in its coagulant function, it had been suggested that the metastatic effect of TF in the severe combined immunodeficient mouse model did not involve products of the coagulation cascade (6). However, this assumption may not be valid since the extracellular TF mutant can bind to VIIa and the resultant TF/VIIa complexes were defective but not completely devoid of the coagulant function (25). Moreover, Fischer *et al.* (26) showed that the thrombin generation is necessary for the TF-dependent hematogenous metastasis (26). In a recent study, Ruf and Mueller (27) provided evidence that TF/VIIa has to be catalytically active for TF-dependent tumor cell metastasis.

In conclusion, our observation that the binding of VIIa to TF induces intracellular signaling was consistent with the earlier observations that VIIa binding is required for TF-mediated signal transduction events such as generation of cytosolic calcium transients (9) and transient tyrosine phosphorylation of several polypeptides (10). Up-regulation of PAP upon VIIa binding to TF suggests that the binding of VIIa to cell surface TF not only initiates the coagulation cascade but also transduces signal(s) that may play an important role in angiogenesis and tumor metastasis.

We acknowledge the contribution of Todd Williams in cell culture. During this investigation, L.V.M.R was a recipient of a Research Career Development Award for the National Heart, Lung, and Blood Institute.

1. Rapaport, S. I. & Rao, L. V. M. (1995) *Thromb. Haemostasis.* **74,** 7–17.
2. Drake, T. A., Morrissey, J. H. & Edgington, T. S. (1989) *Am. J. Pathol.* **134,** 1087–1097.
3. Fleck, R. A., Rao, L. V. M., Rapaport, S. I. & Varki, N. (1990) *Thromb. Res.* **59,** 421–437.
4. Camerer, E., Kolsto, A. B. & Prydz, H. (1996) *Thromb. Res.* **81,** 1–41.
5. Mueller, B. M., Reisfeld, R. A., Edgington, T. S. & Ruf, W. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11832–11836.
6. Bromberg, M. E., Konigsberg, W. H., Madison, J. F. & Pawashe, A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 8205–8209.
7. Zhang, Y., Deng, Y., Luther, T., Muller, M., Ziegler, R., Waldherr, R., Stern, D. M. & Nawroth, P. (1994) *J. Clin. Invest.* **94,** 1320–1327.
8. Zioncheck, T. F., Roy, S. & Vehar, G. A. (1992) *J. Biol. Chem.* **267,** 3561–3564.
9. Rottingen, J. A., Enden, T., Camerer, E., Iversen, J. G. & Prydz, H. (1995) *J. Biol. Chem.* **270,** 4650–4660.
10. Masuda, M., Nakamura, S., Murakami, T., Komiyama, Y. & Takahashi, H. (1996) *Eur. J. Immunol.* **26,** 2529–2532.
11. Rao, L. V. M., Robinson, T. & Hoang, A. D. (1992) *Thromb. Haemostasis* **67,** 654–659.
12. Callander, N. S., Rao, L. V. M., Nordfang, O., Sandset, P. M., Warn-Cramer, B. & Rapaport, S. I. (1992) *J. Biol. Chem.* **267,** 876–882.
13. Liang, P. & Pardee, A. B. (1992) *Science* **257,** 967–971.
14. Liang, P., Averboukh, L. & Pardee, A. B. (1993) *Nucleic Acids Res.* **21,** 3269–3275.
15. Maniatis, T., Fritsch, E. & Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
16. Thuresson, A., Astrom, J. & Gronvik, K. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 979–983.
17. Raabe, T., Bollum, F. J. & Manley, J. L. (1991) *Nature (London)* **353,** 229–234.
18. Aiello, L. P., Robinson, G. S., Lin, Y. W., Nishio, Y. & King, G. L. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6231–6235.
19. Wahle, E., Martin, G., Schiltz, E. & Keller, W. (1991) *EMBO J.* **10,** 4251–4257.
20. Manley, J. (1995) *Curr. Opin. Genet. Dev.* **5,** 222–228.
21. Wahle, E. (1995) *Biochim. Biophys. Acta* **1261,** 183–194.
22. Jackson, R. J. & Standart, N. (1990) *Cell* **62,** 15–24.
23. Wilson, T. & Treisman, R. (1988) *Nature (London)* **336,** 396–399.
24. Ruf, W. & Mueller, B. M. (1996) *Curr. Opin. Hematol.* **3,** 379–384.
25. Ruf, W., Miles, D. J., Rehemtulla, A. & Edgington, T. S. (1992) *J. Biol. Chem.* **267,** 6375–6381.
26. Fischer, E. G., Ruf, W. & Mueller, B. M. (1995) *Cancer Res.* **55,** 1629–1632.
27. Ruf, W. & Mueller, B. M. (1997) *Thromb. Haemostasis* (Suppl), 594 (abstr.).

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 45, Issue of November 5, pp. 32225–32233, 1999
Printed in U.S.A.

Coagulation Factors VIIa and Xa Induce Cell Signaling Leading to Up-regulation of the *egr-1* Gene*

(Received for publication, April 7, 1999, and in revised form, August 30, 1999)

Eric Camerer‡, John-Arne Røttingen‡§, Elisabet Gjernes‡, Kristin Larsen§, Anne Helen Skartlien‡, Jens-Gustav Iversen§, and Hans Prydz‡¶

From the ‡Biotechnology Centre of Oslo and §Laboratory of Intracellular Signaling, Department of Physiology, Institute of Basic Medical Sciences, University of Oslo, N-0371 Oslo, Norway

Intracellular signaling induced by the coagulation factors (F) VIIa and Xa is poorly understood. We report here studies on these processes in a human keratinocyte line (HaCaT), which is a constitutive producer of tissue factor (TF) and responds to both FVIIa and FXa with elevation of cytosolic Ca^{2+}, phosphorylation of extracellular signal-regulated kinase (Erk) 1/2, $p38^{MAPK}$, and c-Jun N-terminal kinase, and up-regulation of transcription of the early growth response gene-1 (*egr-1*). Using *egr-1* as end point, we observed with both agonists that phosphatidylinositol-specific phospholipase C and the mitogen-activated protein kinase/Erk kinase/Erk pathway were mediators of the responses. The responses to FVIIa were TF-dependent and up-regulation of *egr-1* mRNA did not require presence of the TF cytoplasmic domain. Antibodies to EPR-1 and factor V had no effect on the response to FXa. We have provided evidence that TF is not the sole component of the FVIIa receptor. The requirement for proteolytic activity of both FVIIa and FXa suggests that protease-activated receptors may be involved. We now report evidence suggesting that protease-activated receptor 2 or a close homologue may be a necessary but not sufficient component of this particular signal transduction pathway. The up-regulation of *egr-1* describes one way by which the initiation of blood coagulation may influence gene transcription. The ability of these coagulation proteases to induce intracellular signals at concentrations at or below the plasma concentrations of their zymogen precursors suggests that these processes may occur also *in vivo*.

The vitamin K-dependent serine protease clotting factors have traditionally been thought to exert their effects in the fluid phase of the body fluids or at the surface of cells in contact with such fluids. Increasing evidence indicates that activation of these clotting factors also elicits numerous and profound alterations in the biology of the cells on whose surface the activation takes place or where the activated factor is bound. Direct intracellular signaling in cells exposed to the coagulation factor VIIa (FVIIa)[1] was first reported in 1995 (1). It was found to be entirely dependent on the presence of tissue factor (TF), the factor VII- and VIIa -binding trigger of blood coagulation (2) on the cell surface. More recently, FVIIa has been shown to induce phosphorylation of the extracellular signal-regulated kinases 1 and 2 (Erk 1/2) (3) and to up-regulate three mRNA species: poly(A) polymerase (4) and vascular endothelial growth factor (5) in fibroblasts and urokinase type plasminogen activator receptor in pancreatic cancer cell lines (6). Two recent studies demonstrate the involvement of TF in cell adhesion/motility (7, 8). These effects may be mediated by interactions of the TF cytoplasmic tail with cytoskeletal adaptor proteins (7), and probably reflect another aspect of TF biology.

In the case of the coagulation factor Xa (FXa), Gajdusek *et al.* (9) reported induction of release of growth factors from the endothelium. Gasic *et al.* (10), and later Ko *et al.* (11), observed FXa-induced vascular smooth muscle cell proliferation. Signaling was suggested to involve rapid release of platelet-derived growth factor (PDGF), followed by PDGF receptor-mediated activation of the $p21^{ras}/p74^{raf-1}$ pathway (11). We showed that FXa triggered an increase in cytosolic free Ca^{2+} in Madin-Darby canine kidney cells (12). In endothelial cells addition of FXa can activate NO synthase (13, 14) and induce synthesis of cytokines and expression of adhesion molecules (14, 15). Antibodies to PDGF (11, 16, 17) or effector cell protease receptor-1 (EPR-1) (17, 18) both attenuate mitogenic responses to FXa in endothelial cells and in vascular smooth muscle cells. Factor V (FV), the cellular cofactor for FXa in formation of the prothrombinase complex (2), has to our knowledge not been shown to participate in any of these events.

Both FVIIa and FXa must be proteolytically active to induce the signaling process(es) (12). In the case of FVIIa, TF serves as its binding receptor but does not undergo any proteolytic cleavage in the process. This suggests that another molecule is the substrate for cleavage by the indispensable protease activity of FVIIa, and that this cleavage may trigger the intracellular signaling. The presence of TF on the cell surface is, however, an absolute requirement for the FVIIa induced intracellular changes to occur. Our hypothesis is thus that TF efficiently binds FVII/FVIIa present in plasma and other body fluids, and

* This work was supported by grants from the Research Council of Norway, the Norwegian Council on Cardiovascular Diseases, the Norwegian Cancer Society, the Jahre and Owren Foundations, and Rakel and Otto Bruun's Legacy. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Biotechnology Center of Oslo, Gaustadalléen 21, N-0371 Oslo, Norway. Tel.: 47-22-95-87-55; Fax: 47-22-69-41-30; E-mail: hans.prydz@biotek.uio.no.

[1] The abbreviations used are: FVIIa, factor VIIa; $[Ca^{2+}]_c$, cytosolic free Ca^{2+} concentration; DMEM, Dulbecco's modified Eagle's medium; EPR-1, effector cell protease receptor-1; Erk, extracellular signal-regulated kinase; Mek, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; FVIIai, 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone-inhibited FVIIa; FVa, factor Va; FXa, factor Xa, FXai, 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone-inhibited FXa; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBSS, Hepes-buffered salt solution; JNK, c-Jun N-terminal kinase; MDCK, Madin-Darby canine kidney; PAR, protease-activated receptor; PDGF, platelet-derived growth factor; PI-PLC, phosphatidylinositol-specific phospholipase C; PTX, pertussis toxin; MAPK, mitogen-activated protein kinase; TFPI, human tissue factor pathway inhibitor 1; TF, tissue factor; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; RI, relative intensity; MOPS, 4-morpholinepropanesulfonic acid.

presents it in activated form to another transmembrane protein, which is then cleaved to trigger the signaling process(es).

Thrombin has long been known as a powerful cellular activator (19, 20). Several of its effects were recently shown to be mediated through proteolytic activation of one or more of its receptors. All three presently known (PAR1, PAR3, and PAR4) are members of the protease-activated receptor (PAR) family (21–24). We have previously reported data excluding receptors down-regulated by thrombin as candidate substrates for FVIIa or FXa. This paper reports experiments carried out to study PAR2 (22) in this respect.

Using a human keratinocyte line, we have addressed questions concerning the signaling pathway(s) of FVIIa and Xa and some of their cellular consequences, using altered expression of the *egr-1* gene (25) as the end point. This zinc finger transcription factor (Krox-24, NGFI-A, and ZIF/268 are synonyms) showed rapid and transient induction in response to FVIIa and FXa.

EXPERIMENTAL PROCEDURES

Materials

Trypsin, hirudin, ATP, bradykinin, actinomycin D, and Hepes were obtained from Sigma; U73122 and U73343 were from BIOMOL (PA); pertussis toxin (PTX), PD98059, and SB203580 were from Calbiochem (San Diego, CA); keratinocyte SFM with additives, Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, L-glutamine, and fetal calf serum (FCS) were from Life Technologies, Inc. (Paisley, Scotland); recombinant human factor VIIa (FVIIa), DEGRck-inactivated human FVIIa (FVIIai), and recombinant human tissue factor pathway inhibitor (rTFPI) were all kind gifts from Novo-Nordisk (Bagsværd, Denmark); substrate FXa-1 was from Nycomed (Oslo, Norway); purified, RVV-activated human factor X was from Enzyme Research Laboratories (South Bend, IN); the fluorescent calcium indicator fura-2/AM and the surfactant Pluronic F-127 were from Molecular Probes (Eugene, OR); Bacto-dextrose was from Difco; Colorrapid was from LucernaChem (Lucerne, Switzerland); monoclonal mouse anti-human TF (htf1) was kindly donated by Dr. S. Carson (Omaha, NE); alkaline phosphatase-conjugated swine anti-rabbit Ig was from DAKO (Glostrup, Denmark); polyclonal anti-MAPK and anti-phospho-MAPK (Erk 1/2, $p38^{MAPK}$, and JNK) antibodies and monoclonal anti-phospho-Erk 1/2 were from New England Biolabs (Beverly, MA); the anti-EPR-1 monoclonal antibody (B6) was kindly provided by Dr. D. Altieri (Yale University School of Medicine, New Haven, CT); anti-platelet-derived growth factor BB (PDGFBB) was from R&D Systems (Oxon, UK); anti-FV and DEGRck-inactivated human FXa (FXai) were from Hematologic Technologies (Essex Junction, VT); thrombin was kindly provided by Dr. J. W. Fenton II (New York State Department of Health, Albany, NY); the PAR2 agonist peptide SLIGRL (22) was made in our laboratory. Hepes-buffered salt solution (HBSS) consisted of (mM): NaCl, 136; KCl, 5; $MgCl_2$, 1.2; $CaCl_2$, 1.2; Bacto-dextrose, 11; Hepes 10; pH 7.35.

Cell Culture and Transfection

The constitutively TF-expressing keratinocyte line HaCaT (26) was kindly provided by Dr. U. Birk Jensen, Institute of Human Genetics, University of Aarhus, Aarhus, Denmark. HaCaT cells were cultured in keratinocyte-SFM supplemented with recombinant epidermal growth factor (0.5 ng/ml) and bovine pituitary extract (25 μg/ml). After cell detachment during culture, trypsin was inactivated with FCS, which was subsequently removed by repeated washes in keratinocyte SFM. Prior to Northern and Western experiments starvation (2 h prior to agonist addition) and stimulation was done in DMEM without additives. The keratinocyte SFM was low in calcium (<0.1 mM) in order to keep the cells in an undifferentiated state. The change to DMEM prior to stimulation brought the calcium levels up to ~1.8 mM. This change was done to facilitate Ca^{2+}-dependent binding of the clotting factors to TF and possibly other surface receptors, and to allow calcium influx. Great care was taken to establish correct controls for the possible effects of this shift-up.

The human kidney epithelial line HK-2 (ATCC) was cultured as described for the HaCaT cells. CHO cells expressing PAR2 were a kind gift from Johan Sundelin (University of Lund, Lund, Sweden) (27). These were cultured in α-minimal essential medium without nucleosides, supplemented with 10% dialyzed FCS, L-glutamine, antibiotics,

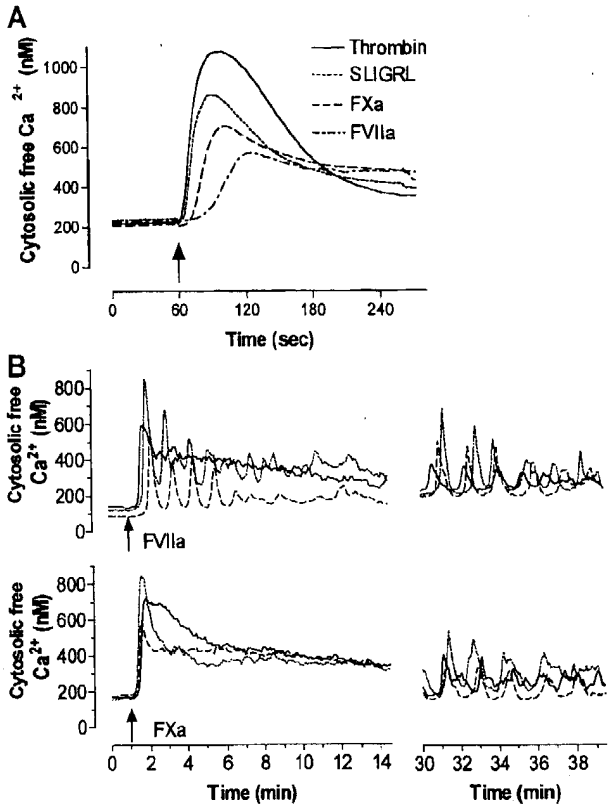


FIG. 1. **Calcium responses in HaCaT cells.** Ca^{2+} oscillations induced by treating HaCaT cells with FVIIa (100 nM), FXa (174 nM), SLIGRL (50 μM) or thrombin (1 unit/ml). *Panel A*, average responses in 330–580 cells. *Panel B*, examples of single cell responses to FVIIa and FXa. Different cells are shown in the 0–15-min and in the 30–40-min time windows. *Arrows* indicate addition of agonist.

and methotrexate (20 nM). COS-1 cells were maintained in DMEM supplemented with 5% inactivated FCS, L-glutamine, and antibiotics.

COS-1 cells were transfected with the mammalian expression vector pcDNA3, or pcDNA3 containing constructs coding for wild type (hTF_{1-263}) or truncated (hTF_{1-246}) human tissue factor cDNA. These constructs have been described in detail previously (28). The truncated construct was identical to wild type except that the sequence coding for the last 18 C-terminal amino acids had been deleted. The PAR2-expressing CHO-DG44 cells were transfected with hTF_{1-263} only. Transfection was done by standard calcium phosphate coprecipitation procedures with glycerol shock. Cells with stably integrated constructs were selected with 500 μg/ml G418. TF expression was confirmed by total activity measurement in cell homogenates (28). Surface TF activity was determined by a FXa chromogenic substrate assay (28). Immunofluorescence was carried out on ethanol-fixed cells with htf1 and propidium iodide costaining (28).

Isolation of mRNA and Northern Blot Analysis

In these experiments medium was always changed to DMEM without additives for 2 h prior to addition of agonist to the cells. Unless otherwise stated, pretreatment with inhibitor or vehicle was done in this 2-h period. Agonist or vehicle was made up to 20% of the final volume, preheated to 37 °C, and added gently to the cells. Cells were harvested by washing in ice-cold PBS, then scraped off into 0.4 ml of lysis buffer (100 mM Tris-HCl, pH 8, 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM dithiothreitol) and sheared with a 21-gauge syringe. For isolation of mRNA, oligo(dT)-conjugated magnetic beads (Dynal, Oslo, Norway) were used according to the manufacturer's instructions. Samples were run on agarose/formaldehyde gels in MOPS buffer and blotted. Prehybridization and hybridization were performed in ExpressHyb™ solution from CLONTECH. Complete cDNAs were used to generate probes for *egr-1*, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and TF as described by Feinberg and Vogelstein (29, 30). The oligonucleotide probe used to detect the TF cytoplasmic domain had the sequence 5′-GTGGGGAGTTCTCCTTCCAGCTCTGCCCCACTCCTGCC-

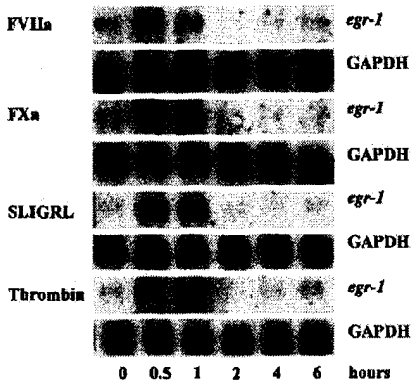


FIG. 2. **Up-regulation of *egr-1* mRNA in HaCaT cells.** Changes in *egr-1* mRNA levels with time in response to FVIIa (100 nM), FXa (174 nM), SLIGRL (50 μM), or thrombin (1 unit/ml) in HaCaT cells. Northern blots were hybridized with ^{32}P-labeled probes for *egr-1* and GAPDH. The results are representative of three independent experiments.

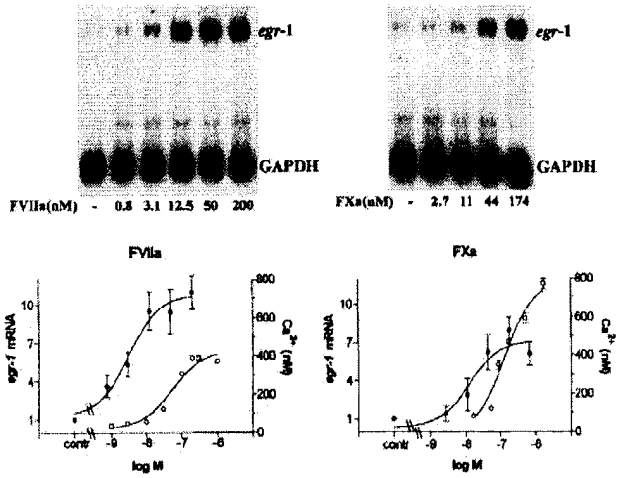


FIG. 3. **Effects of various concentrations of FVIIa and FXa on induced levels of *egr-1* mRNA and $[Ca^{2+}]_c$ in HaCaT cells.** *Upper panels* show the effect of increasing concentrations of FVIIa and FXa on levels of *egr-1* mRNA. Cells were harvested 45 min after agonist addition. The *lower panels* show average responses with their standard errors in logarithmic plots. The *egr-1* intensities were normalized to GAPDH intensities for the same sample and further normalized to cells treated with vehicle alone. For the *egr-1* data (*closed circles*), each point is based on four independent mRNA isolations from cells harvested 45 min after agonist addition. Concentration-dependent increases in maximal Ca^{2+} response during the first 210 s after addition of protease are plotted in the same graph (*open circles*, n = 65–280). Results are presented as average values and their standard error.

3′. For reprobing, the filters were stripped in 0.5% SDS at 100 °C for 5–10 min. Quantitation was done using a PhosphorImager (Molecular Dynamics).

Immunoblotting

Cells were washed as for mRNA isolation, and lysed directly in a reducing SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% bromphenol blue). Cell lysates were sonicated to shear DNA, denatured for 5 min at 100 °C and resolved by SDS-polyacrylamide gel electrophoresis, blotted onto Immobilon P membranes (Millipore), and the membranes blocked for 1 h in Tris-buffered saline with Tween (10 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween) with 5% BSA. Immunoblotting with anti-phospho-MAPK or anti-MAPK antibodies (anti-Erk 1/2, anti-p38^{MAPK}, and anti-JNK, 1/1000, overnight) followed by an alkaline phosphatase-conjugated swine anti-rabbit secondary antibody (1/1000, 1 h) was done at 4 °C. After repeated washes with Tris-buffered saline with Tween, the membranes were developed with Vistra ECF (Amersham Pharmacia Biotech) and quantitated using a PhosphorImager. Identical amounts of

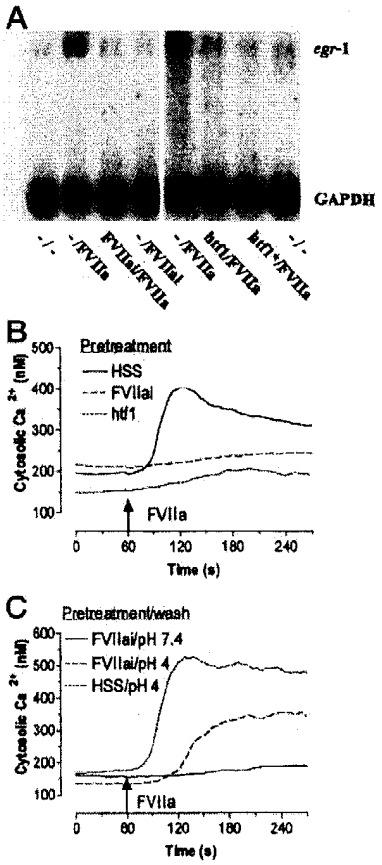


FIG. 4. **Effect of antibody to TF and of inactivated FVIIa on induction of *egr-1* mRNA and $[Ca^{2+}]_c$ increases in HaCaT cells.** *Panel A*, cells were pretreated for 2 h with DMEM (vehicle) (−), FVIIai (100 nM), or htf1 (5 or 25 μg/ml) prior to addition of vehicle (−), FVIIa (50 nM), or FVIIai (100 nM) as indicated. Cells were harvested for mRNA isolation 45 min after agonist addition. Northern filters were hybridized with ^{32}P-labeled probes to *egr-1* and GAPDH as described. *Panel B*, cells (100–130) were pretreated with HBSS (vehicle), FVIIai (100 nM), or htf1 (5 μg/ml) for 45 min, washed, and FVIIa (100 nM) added 60 s later (*arrow*). *Panel C*, cells (70–100) were pretreated with HBSS or FVIIai (100 nM) for 45 min, washed at pH 4.0 or 7.4 as indicated, and FVIIa (100 nM) added at 60 s (*arrow*).

the same extracts were run in parallel for immunoblotting with anti-phospho-MAPK antibody and anti-MAPK antibody.

Measurement of $[Ca^{2+}]_c$ in Single Cells

Measurement of $[Ca^{2+}]_c$ in single cells was done as described previously (1). Cells were incubated for 45 min at 37 °C with a solution of 5 μM fura-2, 0.25% Me_2SO and 0.005% Pluronic F-127 in HBSS. Pretreatments were within the 45-min fura-2 loading period, or 16–20 h prior to this in the case of PTX. After fura-2 loading, the cells were washed twice and incubated with 400 μl of HBSS. Additions to the cell cultures were done by injection of 100 μl into the well. Injection of the agonist vehicle was used in the control cultures. The Ca^{2+} imaging and registration software has been developed in our laboratory (31). The cytosolic Ca^{2+} concentration was calculated using the equation $[Ca^{2+}] = K_d\ \beta\ (R - R_{min})/(R_{max} - R)$ (32). Calibrations were done as described previously (33). The experiments were carried out at 37 °C.

Quantitative Analysis

Calcium—The maximum and average Ca^{2+} increases were calculated as the difference between the values after application of agonist and the average Ca^{2+} levels before. The average reflects the integral of the Ca^{2+} response. Cells with spontaneous responses before addition of agonist (ranging from 0 to 13% in different experiments) were excluded by not considering cells that during the first 60 s of observation had either a higher absolute Ca^{2+} level than 400 nM or a difference of more than 75 nM between the maximal and minimal Ca^{2+} levels. All other cells, both responders and non-responders, were included in the calcu-

TABLE I

Effect of extracellular inhibitors on increased levels of $[Ca^{2+}]_c$ and egr-1 mRNA induced by FVIIa and FXa in HaCaT cells

Inhibition was calculated as described under "Experimental Procedures." In each experiment agonist response without the inhibitor was taken as 100%. For $[Ca^{2+}]_c$, calculations were based on the average Ca^{2+} values during the first 210 s after addition of agonist; cells were washed after pretreatment. Pretreatment did not affect the percentage of excluded cells. For *egr-1* mRNA, no wash was introduced between pretreatment and agonist addition; cells were harvested 15 min after addition of agonist. RNA blots were hybridized with *egr-1* and GAPDH probes.

Pretreatment		Agonist							
		FVIIa (100 nM)				FXa (174 nM)			
		Ca^{2+}		*egr-1*		Ca^{2+}		*egr-1*	
		Inhibition ± S.E. (%)	*n*	Inhibition ± S.E. (%)	*n*	Inhibition ± S.E. (%)	*n*	Inhibition ± S.E. (%)	*n*
FVIIai	100 nM	87 ± 4**[c]	105	95 ± 5**	10	−22 ± 5 (NS)	105	16 ± 24 (NS)	4
FXai	174 nM	−7 ± 7 (NS)	66			−14 ± 4 (NS)	103	8 ± 21 (NS)	4
htf-1	25 μg/ml	83 ± 3**	100	93 ± 3**	6	9 ± 8 (NS)	54		
B6	50 μg/ml[a]					−1 ± 5 (NS)	72	9 ± 13 (NS)	3
Anti-PDGFBB	10 μg/ml							11 ± 10 (NS)	4
Anti-FV	10 μg/ml					24 ± 3 (NS)	196	4 ± 6 (NS)	4
rTFPI[b]						91 ± 5**	72	109 ± 3**	4
Hirudin	5 units/ml					14 ± 4 (NS)	211	17 ± 9 (NS)	6

[a] 10 μg/ml B6 was used in mRNA studies.
[b] rTFPI was preincubated with FXa at 3 times molar excess before addition.
[c] *, $p < 0.05$; **, $p < 0.01$; NS, not significant.

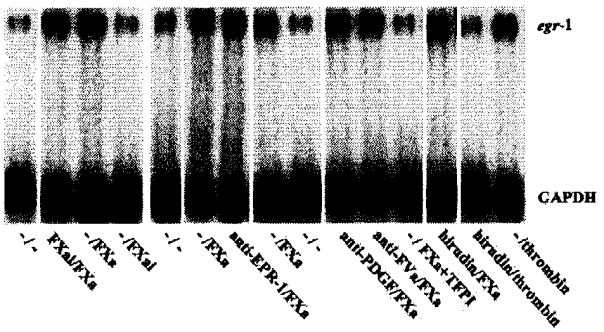


FIG. 5. **Effect of some inhibitors and antibodies on the induction of *egr-1* mRNA by FXa.** Cells were pretreated (2 h) with DMEM (vehicle) (−), FXai (174 nM), B6 (anti-EPR-1; 10 μg/ml), anti-PDGFBB (10 μg/ml), anti-FVa (10 μg/ml), or hirudin (5 units/ml) prior to addition of vehicle (−), FXa (87 nM), a preincubated 3:1 mixture of TFPI and FXa (87 nM), FXai (174 nM), and thrombin (0.25 unit/ml) as indicated. Cells were harvested for mRNA isolation 45 min after agonist addition. Northern filters were hybridized with ^{32}P-labeled probes to *egr-1* and GAPDH. Quantitation of data in Table I.

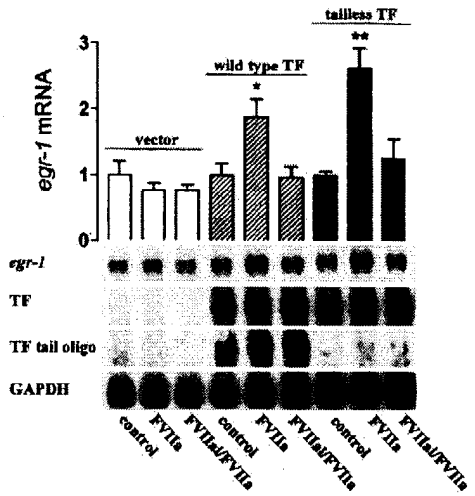


FIG. 6. **FVIIa-induced *egr-1* up-regulation in TF-transfected COS-1 cells.** COS-1 cells were transfected to express TF (wild type or truncated Δ245). Controls were transfected with empty vector. Stable transfectants were pretreated with DMEM (vehicle) (−) or FVIIai (100 nM) for 2 h prior to addition of vehicle (−) or FVIIa (50 nM). Cells were harvested for mRNA isolation 45 min after agonist addition. The same blot was hybridized with ^{32}P-labeled probes to *egr-1*, TF, and GAPDH, and after stripping, with an oligonucleotide probe corresponding to the sequence deleted from the TF cytoplasmic domain. The *bars* (*upper part*) show the average increase of *egr-1* mRNA relative to GAPDH with their standard errors from four experiments.

lations of the Ca^{2+} signals. The data are presented as means and their standard error (S.E.). Statistical significance was tested using a general mixed model analysis of variance (3V module BMDP).

mRNA—After subtraction of average background, intensity data obtained from the PhosphorImager for *egr-1* mRNA were normalized to GAPDH mRNA intensities for all samples. -Fold induction of *egr-1* mRNA was determined by setting to one the relative intensity (RI) in cells treated with vehicle only. Percentage of inhibition was calculated by the following formula: percentage of inhibition = 100 − 100 × ((RI of the given sample − RI of vehicle control)/(RI of normal agonist response − RI of vehicle control). Thus, a stimulatory effect will appear as a "negative inhibition," and a reduction below basal levels of *egr-1* will appear as a greater than 100% inhibition. Data are presented as means and their standard error (S.E.). Significance was tested using a two-tailed Student's *t* test with Welch's correction. A single asterisk (*) indicates a *p* value below 0.05, whereas a double (**) indicates a *p* value below 0.01. NS, not significant.

Phosphoprotein—Intensity data obtained from the PhosphorImager for the phosphorylated form of MAPKs were normalized to total MAPK intensities from identical amounts of the same samples run and blotted in parallel. -Fold increase in phospho-MAPK was determined by setting the RI in vehicle-treated cells to 1. Statistical analysis was as for *egr-1* mRNA.

RESULTS

Choice of Cell Types—We have previously reported that FVIIa triggers Ca^{2+} responses in MDCK cells, in COS-1 cells transfected to express TF, in J82 cells and in human umbilical vein endothelial cells (HUVEC) (1), and that FXa induced responses similar to FVIIa in MDCK cells (12). The signals varied between cell types and between individual cells within a population (1, 12). The response rate in MDCK was close to 100% (1, 12), but their canine origin served to limit our access to antibodies and other useful reagents. The spontaneously immortalized, non-tumorigenic keratinocyte human cell line (HaCaT) is a high level constitutive producer of TF. HaCaT cells responded with increased Ca^{2+} levels to both FVIIa and FXa, as well as to thrombin and SLIGRL, showing that this line also expresses PAR2 and thrombin receptors (Fig. 1, *panel A*). FVIIa and FXa both triggered a sustained elevation of $[Ca^{2+}]_c$ (Fig. 1, *panel B*) in almost all cells tested. Their response pattern differed from that of MDCK cells (12). In HaCaT cells the Ca^{2+} oscillations were less prominent and non-synchronous and were replaced by a continuous elevation of $[Ca^{2+}]_c$, generally failing to reach baseline during the initial minutes of response. The responses lasted approximately as long as the

TABLE II
Desensitization of Ca^{2+} responses in HaCaT cells

Cells were pretreated (45 min) with FVIIa (200 nM), FXa (348 nM), SLIGRL (200 μM), thrombin (1 unit/ml), trypsin (2 units/ml), or bradykinin (100 nM). After washing, FVIIa (100 nM), FXa (178 nM), SLIGRL (50 μM), thrombin (1 unit/ml), or bradykinin (100 nM) were added at 60 s. Calculations are based on the average level of $[Ca^{2+}]_c$ in the first 210 s after agonist addition. The agonist response without pretreatment within the same experiment was taken as 100% and used as control for statistical comparison. In these experiments approximately 10% of the cells were excluded from the calculations because of spontaneous responses before addition of agonists (see "Experimental Procedures"). With some pretreatments, *e.g.* FVIIa, this percentage was higher (around 20%) because of a continuing response caused by the pretreatment. The number of individual cells examined ranged from 100 to 500.

Pretreatment	Inhibition (%) ± S.E.				
	FVIIa	FXa	SLIGRL	Thrombin	Bradykinin
HSS	0 ± 8[a]	0 ± 2	0 ± 2	0 ± 4	0 ± 2
FVIIa	99 ± 5**	69 ± 2**	−18 ± 7 (NS)	−20 ± 7 (NS)	ND
FXa	91 ± 2**	99 ± 1**	59 ± 2**	63 ± 3**	ND
SLIGRL	57 ± 2**	58 ± 2**	88 ± 1**	37 ± 3 (NS)	ND
Thrombin	7 ± 5 (NS)	−21 ± 3 (NS)	ND	95 ± 1**	ND
Bradykinin	16 ± 4 (NS)	−6 ± 2 (NS)	19 ± 7 (NS)	ND	67 ± 2**
Trypsin	81 ± 1**	98 ± 1**	74 ± 2**	81 ± 2**	−22 ± 3 (NS)

[a] See Footnote *c* to Table I. ND, not determined.

MDCK cell responses (12), although this has not been explored in detail. If not otherwise stated, the experiments reported have been carried out with the HaCaT cell line.

Transcriptional End Point—To facilitate dissection of the receptor/signaling system(s) engaged by FVIIa and FXa, we looked for a rapidly expressed, abundant mRNA regulated by these factors. The early growth response gene *egr-1* showed rapid (30–60 min), transient (back to basal level within 2 h) and pronounced (10-fold) up-regulation of mRNA levels in response to FVIIa and FXa, as well as to SLIGRL and thrombin (Fig. 2). Inhibition of transcription by actinomycin D (10 μg/ml) added 5 min prior to stimulation abolished the induction of *egr-1* by both proteases ($p < 0.001$; data not shown). The increase in *egr-1* mRNA levels is thus highly likely to be a result of induced transcription rather than mRNA stabilization. This is the first demonstration of a transcription factor induced by these two clotting factors.

Both $[Ca^{2+}]_c$ and *egr-1* mRNA responses were concentration-dependent. EC_{50} values were an order of magnitude lower for the *egr-1* mRNA response than for the calcium response (3 and 54 nM, respectively for FVIIa; 13 and 130 nM, respectively, for FXa) (Fig. 3). Both proteases gave a maximum *egr-1* response at concentrations below the plasma concentrations of the corresponding zymogen precursor (10 nM for FVII and 178 nM for FX) (34).

The Ca^{2+} responses in MDCK cells to FVIIa and FXa were previously shown to be specific (*i.e.* not caused by contaminants in the agonist preparations) and to require their intact proteolytic activity (12). These experiments were repeated and expanded to ascertain that the Ca^{2+} and *egr-1* mRNA responses seen in the HaCaT cells were specifically caused by the same factors in their activated state. Active site-inhibited FVIIa (FVIIai) did not induce responses in $[Ca^{2+}]_c$ (data not shown) or *egr-1* mRNA (Fig. 4, *panel A*; Table I). A marked inhibition of both *egr-1* up-regulation and Ca^{2+} increase was seen when pretreating the cells with a neutralizing monoclonal antibody to TF (htf1) (Fig. 4, *panels A* and *B*; Table I). This excluded involvement of endotoxin and other contaminants, as well as demonstrating also in the HaCaT cell line the absolute requirement for TF and for proteolytic activity of the agonists. Pretreatment of the cells with FVIIai inhibited the effect of FVIIa. This inhibition was to a large extent reversible when cells were washed at pH 4 to remove FVIIai (Fig. 4, *panel C*) (28).

The binding site for FXa on HaCaT cells is unknown. The two main cellular components known to bind FXa are EPR-1 and FVa. Antibodies expected to block FXa binding to either of these were tested, and none had any effect on the *egr-1* mRNA or $[Ca^{2+}]_c$ changes in response to FXa (Fig. 5, Table I). Neither

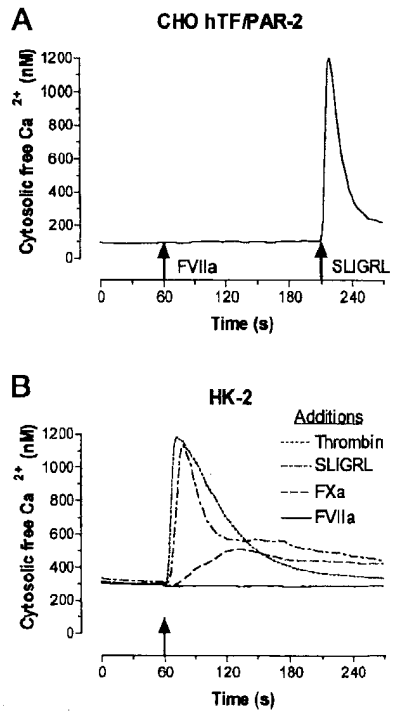


FIG. 7. **Absence of Ca^{2+} responses to FVIIa in CHO and HK-2 cells expressing PAR2 and tissue factor.** *Panel A*, CHO cells stably transfected to express PAR2 and tissue factor. *Arrows* indicate time of addition of FVIIa (final concentration 200 nM) and SLIGRL (100 μM). Average of 100 cells is shown. *Panel B*, Ca^{2+} responses to FVIIa and FXa in HK-2 cells expressing PAR2 and tissue factor. Panel shows average Ca^{2+} responses to FVIIa (200 nM), FXa (174 nM), SLIGRL (100 μM), and thrombin (1 unit/ml) in HK-2 cells pretreated for 4 h with tumor necrosis factor-α to increase TF expression (80–150 cells). *Arrow* indicates time of additions.

rPDGFBB directly (data not shown) nor antibodies to PDGFBB had any effect in our system (Fig. 5, Table I). HaCaT cells may not express PDGF receptors. Active site-inhibited FXa (FXai) did not induce responses in $[Ca^{2+}]_c$ or *egr-1* mRNA (Fig. 5, Table I). In contrast to the effect of FVIIai on the response to FVIIa, pretreatment with FXai had no inhibitory effect on a subsequent incubation with FXa (Fig. 5, Table I), either when the cells were washed at pH 7.4 prior to addition of FXa (Ca^{2+} response) or when left unwashed (*egr-1* response).

Specificity was demonstrated by the fact that pretreatment of FXa with a recombinant preparation of human tissue factor

pathway inhibitor (rTFPI), which is the main physiological inhibitor of FXa, abolished both $[Ca^{2+}]_c$ and *egr-1* mRNA responses (Fig. 5, Table I). TFPI is also an inhibitor of FVIIa, so the effects of FXa could still be attributable to contaminating FVIIa in the FXa preparation. FXa stimulation, however, was unaffected by preincubation of the cells with FVIIai, which would block the response to contaminating FVIIa (Table I). Finally, hirudin was used to exclude generation of trace amounts of thrombin as a source for the activation (Fig. 5, Table I).

Transducing Receptors—The active site requirement of FVIIa and the lack of proteolysis of TF when in complex with FVIIa suggest that the receptor may be more complex. A dual receptor system with one binding and one proteolytically activated transducing receptor may seem likely. The lack of direct correlation between TF expression and FVIIa responsiveness seen in CHO and HK-2 cells supports this view (see below). If TF was not directly involved in signaling but rather in binding of the agonist, one might expect removal of the cytoplasmic tail of TF to have little or no effect. Extracellular binding of FVIIa should be unaltered (12, 35). We therefore compared the *egr-1* up-regulation in response to FVIIa in COS-1 cells transfected with a construct encoding wild type human TF (hTF_{1-263}), a construct encoding human TF lacking the 18 C-terminal amino acids (hTF_{1-245}, "tailless" TF) or vector control. The *egr-1* response in cells expressing the tailless TF construct was at least as high as in cells expressing wild type TF (Fig. 6). Vector-transfected cells, which had very low TF activity, did not respond. Hybridization of the same Northern blots with an oligonucleotide probe corresponding to the sequence deleted from TF confirmed that the cells did indeed express tailless TF (Fig. 6). For both TF constructs only a limited number (10–20%) of the transfected cells expressed TF, which probably explains the low level of *egr-1* mRNA increase relative to that seen in HaCaT cells.

The four cloned PARs are candidate transducing receptor components for both FVIIa and FXa. We have previously excluded the candidacy of receptors that can be down-regulated by thrombin in MDCK cells. This leaves PAR2 as the only presently known candidate for being the FVIIa or FXa protease-activated receptor. PAR2 is activated by trypsin and by mast cell tryptase and may have additional, unidentified activators in the vasculature (36). Factor Xa has been reported to activate PAR2 to some degree (37). In heterologous desensitization of the Ca^{2+} responses (Table II), pretreatment with low concentrations of trypsin or with the PAR2 agonist SLIGRL for 45 min down-regulated the response to FVIIa by 81% and 57%, respectively. The FXa effect was similarly reduced by 98% and 58%. Trypsin inhibited all the protease-inducible Ca^{2+} signals tested, but not the response to bradykinin (Table II). Under the conditions used, trypsin treatment did not reduce cell surface TF (data not shown). Taken together, these results indicated the trypsin sensitivity of the signaling receptors for FVIIa and FXa. However, various cell surface substrates may have been cleaved by trypsin, which makes these data difficult to interpret. In addition, the desensitization by preincubation with SLIGRL may suggest that PAR2 or a close homologue was involved (Table II).

The question of a role for PAR2 was therefore approached more directly in a transfected CHO cell model. Neither FVIIa nor SLIGRL had any effect on the Ca^{2+} levels of untransfected CHO cells.

PAR2-transfected CHO cells were highly responsive to the PAR2 peptide agonist but gave no significant Ca^{2+} response to the two coagulation factors. The construct $pcDNA3hTF_{1-263}$ was then transfected into CHO cells expressing PAR2. The resulting cells expressed the PAR2 receptor, and all cells responded well to SLIGRL. In addition, more than 50% of the cells expressed high amounts of TF on their surface, fully active in triggering blood coagulation. These cells thus carried high levels of functional TF as well as PAR2, but gave no Ca^{2+} signal when exposed to FVIIa or FXa (Fig. 7*A*). In parallel experiments human HK-2 cells were shown to respond to the SLIGRL peptide (and thus to carry PAR2), to thrombin and (less pronounced) to FXa, but not at all to FVIIa, although the HK-2 line also expresses TF (Fig. 7*B*). Increasing TF expression by induction with tumor necrosis factor-α did not alter the result. Thus, two different cell lines, both carrying PAR2 and TF on their surfaces, were unresponsive to FVIIa, clearly demonstrating that TF and PAR2 are not sufficient for this response. The desensitization of the FVIIa response by SLIGRL suggested that PAR2 or a close homologue may be involved, although factor VIIa did not down-regulate the SLIGRL response. In signaling triggered by FXa desensitization experiments suggested a possible involvement of PAR2 or a close homologue, but PAR2 alone is clearly not sufficient to mediate this signal.

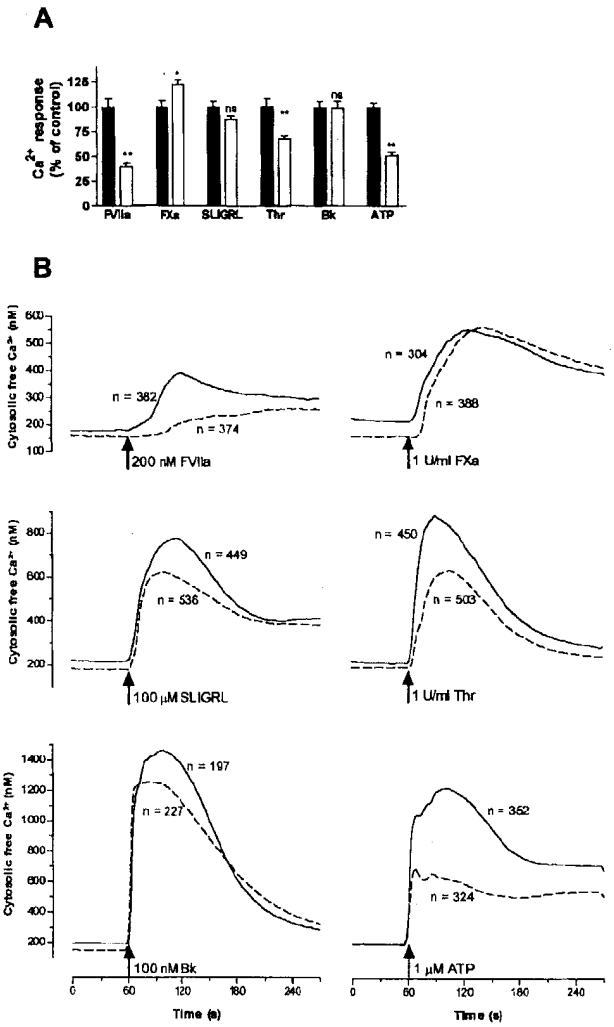


FIG. 8. **Effect of PTX on Ca^{2+} responses to various agonists in HaCaT cells.** *Panel A*, average Ca^{2+} increase in the first 210 s after agonist addition. Response in controls was taken as 100%. *Closed bars*, controls. *Open bars*, cells pretreated for 16 h with 500 ng/ml PTX. Agonists are as in *panel B*. *Panel B*, average Ca^{2+} responses to FVIIa (200 nM), FXa (174 nM), SLIGRL (100 μM), thrombin (1 unit/ml), bradykinin (100 nM), and ATP (1 μM) in cells pretreated with PTX as in *panel A*. *Arrows* indicate additions. *Broken line*, sample pretreated with PTX. *n* = number of cells examined.

TABLE III

Effects of various intracellular inhibitors on FVIIa- and FXa-induced up-regulation of egr-1 mRNA in HaCaT cells

Agonist response without pretreatment within the same experiment was taken as 100%. Unless otherwise stated, cells were preincubated with inhibitor for 2 h (16–20 h for PTX) prior to addition of FVIIa (50 nM) or FXa (87 nM). For mRNA isolation cells were harvested 45 min after agonist addition. RNA blots were hybridized with *egr-1* and GAPDH probes. Intensity values for *egr-1* mRNA were normalized to values obtained for GAPDH before calculating the percentage of inhibition. n = number of independent mRNA isolations.

Inhibitor		Agonist			
		FVIIa		FXa	
		Inhibition ± S.E. (%)	n	Inhibition ± S.E. (%)	n
PTX	500 ng/ml	−22 ± 15 (NS)[a]	4	−29 ± 15 (NS)	3
U73122	5 μM	72 ± 11**	8	87 ± 7**	7
SB203580	20 μM	−24 ± 14 (NS)	4	−49 ± 11*	4
PD98059	1 μM	−1 ± 5 (NS)	6	−25 ± 8*	6
	5 μM	26 ± 8 (NS)	6	−5 ± 13 (NS)	6
	50 μM	93 ± 4**	6	94 ± 8**	6

[a] See Footnote *c* to Table I.

Acting on the circumstantial evidence that the signaling receptor components for FVIIa and FXa belonged to the protease-activated subfamily of G protein-coupled receptors, the effect of pertussis toxin on the responses to the coagulation factors was compared with the effects on signals induced by the other agonists: thrombin, SLIGRL, bradykinin, and ATP. Significantly reduced but not abolished Ca^{2+} signaling was seen with ATP, thrombin, and SLIGRL when HaCaT cells were incubated with PTX (500 ng/ml) for 16–20 h prior to agonist addition (Fig. 8, *A* and *B*). The response to FXa was not altered, whereas the response to FVIIa was markedly reduced over the first few minutes, indicating that these two factors utilize different signaling receptors. $[Ca^{2+}]_c$ in the cells treated with PTX and FVIIa reached the level of control cells (treated with agonist only) after 3–4 min and remained elevated. In Northern blots of *egr-1* mRNA, essentially no differences caused by pretreatment with PTX were observed (Table III), showing that the initial part of the Ca^{2+} signal was not necessary for the *egr-1* response.

Intracellular Signaling Pathways Activated by FVIIa and FXa—Signaling pathways activated upon binding of FVIIa and FXa are insufficiently described. Besides our work on Ca^{2+} signaling (1, 12), phosphorylation of Erk 1/2 has been observed (3). Addition of agonist, be it FVIIa or FXa, leads within seconds to Ca^{2+} release through a mechanism mediated by phosphatidylinositol-specific phospholipase C (PI-PLC) in both MDCK (12) and HaCaT cells (data not shown), as evidenced by the effect of its commonly used but not entirely specific inhibitor U73122 (38). Its most important unspecific effect is to cause release of Ca^{2+} from intracellular stores (39). No such effect was seen in our experiments. The Ca^{2+} signal most probably lies on the pathway to induction of *egr-1* expression, since U73122 rapidly inhibited also this end point (Table III). We confirmed in HaCaT cells the earlier report of the phosphorylation of Erk 1/2 by FVIIa (3) (Fig. 9). In contrast to that report (3), we also observed increased phosphorylation of key components of two other MAPK pathways investigated ($p38^{MAPK}$ and C-Jun N-terminal kinase (JNK), Fig. 9). In addition, FXa induced increased phosphorylation in the same three MAP kinases (Fig. 9). In all cases preincubation with FVIIai abrogated the phosphorylation induced by FVIIa. An extra 44-kDa band seen in the phospho-JNK (p-JNK) blot was probably pErk 1, as it was absent in samples pretreated with PD 98059 (Fig. 9), and its detection in immunoblots was blocked by a monoclonal antibody to pERK (data not shown). Consistent with a role for Erk 1/2 phosphorylation in the signaling leading to enhanced *egr-1* transcription, inhibition of Erk kinases Mek 1/2 by PD 98059 (Fig. 9) also inhibited up-regulation of *egr-1* mRNA by both coagulation factors (Table III). Another inhibitor, SB 203580, with specificity for $p38^{MAPK}$, did not inhibit the *egr-1* response to either factor (Table III).

DISCUSSION

We have previously demonstrated that coagulation factors VIIa and Xa induce intracellular Ca^{2+} signals in various cell types, although not in all (1, 12). Signal transduction initiated by these factors is poorly understood. Most of our previous work was carried out using a MDCK cell line, which, being of canine origin, limited the access to useful antibodies and other reagents. In initial experiments we therefore screened various human cell lines and found that the constitutively TF- producing keratinocyte cell line HaCaT responded with marked Ca^{2+} elevation when exposed to FVIIa or FXa.

Using this cell line we have confirmed the absolute requirement of TF for FVIIa-induced signaling as well as the absolute necessity for both factors being in their proteolytically activated state. We then proceeded to establish a new end point for studies of the transduction pathway(s) in that we discovered that mRNA for the transcription factor Egr-1 was markedly (up to 12-fold) up-regulated when HaCaT cells were exposed to FVIIa or FXa. This is the first description of a link between regulation of a transcription factor and the initiation of the clotting cascade. The increase of the level of *egr-1* mRNA required the same conditions as the Ca^{2+} response (*i.e.* FVIIa and FXa in their proteolytically active state, and an absolute requirement for availability of TF binding in the case of FVIIa). The difference in the EC_{50} values of the clotting factors for the two responses (Fig. 3), being approximately an order of magnitude lower for the *egr-1* response, may be explained by the different time windows of observation. For both proteases (FVIIa and FXa), the EC_{50} values for the *egr-1* response were well below the levels of the corresponding circulating (unactivated) clotting factors in plasma. The average *egr-1* mRNA level induced by FVIIa was higher than that induced by FXa, for the Ca^{2+} signals the opposite was the case, and the FXa-induced response did not plateau.

Using the Ca^{2+} response and up-regulation of *egr-1* mRNA as end points, we addressed the questions of what receptors were engaged by the two proteases and what signaling pathways they activated. TF is required for FVIIa signaling (1, 12), and we have suggested that TF acts as a cofactor, concentrating FVII/FVIIa at the cell surface, rather than as a signal-transducing receptor. The evidence for this has been the lack of Ca^{2+} signals in several cell lines that express TF constitutively and the lack of ability of active site inhibited FVIIa to induce signals when binding to TF. FVIIai binds TF with even higher affinity than FVIIa. We show here that deletion of the C-terminal 18 amino acids from the 21-amino acid-long cytoplasmic tail of TF did not impair FVIIa-induced *egr-1* up-regula-

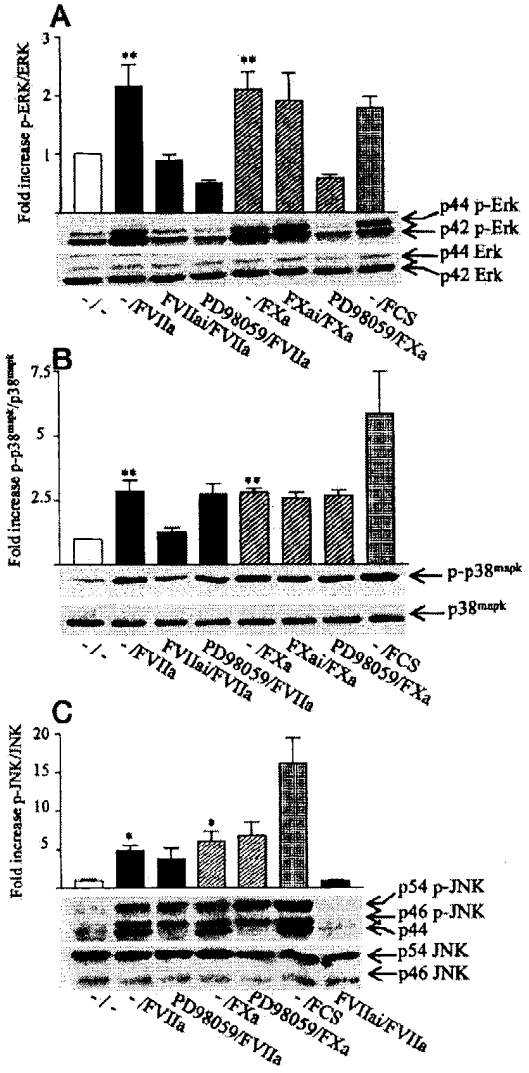


FIG. 9. **FVIIa- and FXa-induced phosphorylation of different MAP kinases in HaCaT cells.** *Panel A* (p-Erk/Erk) and *panel B* (p-p38^{MAPK}/p38^{MAPK}); cells were pretreated for 2 h with DMEM (vehicle) (−), FVIIai (100 nM), FXai (174 nM), or PD98059 (50 μM) prior to addition of DMEM (control), FVIIa (50 nM), FXa (87 nM), or FCS (20%). The cells were harvested in reducing SDS sample buffer 10 (Erk 1/2 and p38^{MAPK}) or 30 (JNK) minutes after agonist addition. Cell extracts were resolved by SDS-polyacrylamide gel electrophoresis, and gels run in parallel were immunoblotted with antibodies to phosphorylated or total MAPK species as indicated. *Panel C* (p-JNK/JNK), cells were treated as in *panels A* and *B* except that pretreatment with FXai was omitted. Please note that succession of lanes in *panel C* differs from *panels A* and *B*. *Bars* represent the average increases and their standard errors in phospho-MAPK relative to total MAPK from four to seven independent experiments. Statistical significance is indicated for FVIIa and FXa additions to untreated cells, and is based on the increase relative to vehicle controls.

tion, suggesting that the TF cytoplasmic domain is not involved in this signaling pathway, thus supporting the need for an additional receptor component.

Whereas a role for TF in binding FVIIa is well established, there is little consensus about FXa binding components or signaling receptors. Antibodies to EPR-1 and to FV at high concentrations had no significant effect on the end point responses. Northern blots with double-stranded cDNA probes to EPR-1 showed only the survivin transcript, which according to the current theory of EPR-1 regulation (40) should exclude the presence of EPR-1 in the HaCaT cells.

In search for signaling receptors activated by FVIIa or FXa, the PARs were evident candidates, being the only receptors known to be activated by proteolysis. Heterologous desensitization experiments with the PAR agonists thrombin, trypsin, and SLIGRL in HaCaT cells confirmed our previous experiments in MDCK cells (12), demonstrating that receptors down-regulated by thrombin (PAR1, -3, and -4) were not involved. Both PAR2 agonists (trypsin and SLIGRL) desensitized HaCaT cells to the effect of FVIIa and FXa while leaving TF intact, thus demonstrating the trypsin sensitivity of the putative signaling receptors. The facts that FXa has a moderate effect on PAR2 and that SLIGRL has an effect on the putative FXa signaling receptor suggest that there may be structural similarities between these two receptors.

However, in direct transfection experiments, the line of CHO cells carrying functional PAR2 as well as TF on their surface did not respond to FVIIa. Similar results were obtained using HK-2 cells, which express PAR2 and TF constitutively. Unless there are even more components to the initial binding/signaling receptor complex, we conclude that the signaling receptors are not yet found.

We have previously shown in MDCK cells, and confirmed in HaCaT cells here (data not shown) that the PI-PLC inhibitor U73122 completely abrogated the $[Ca^{2+}]_c$ changes in response to either of the proteases. We show here that this compound strongly inhibited the *egr-1* response as well, indicating that PI-PLC is a common mediator of the two responses (Table III), and consistent with the *egr-1* response being on the same pathway and downstream of the Ca^{2+} signal. It has been reported (3), and we confirm here, that in certain cell types Erk 1/2 become phosphorylated when FVIIa binds to TF. We expand this observation by showing that exposure of HaCaT cells to FXa also leads to phosphorylation of Erk 1/2, and that both proteases induce significant phosphorylation of p38^{MAPK} and JNK (Fig. 9). Induction of *egr-1* by either of the two coagulation factors was abrogated by PD 98059 (Table III), an inhibitor of the Erk 1/2 kinases Mek 1/2. An inhibitor of p38^{MAPK}, SB203580, had essentially no effect. This suggests a route to *egr-1* induction via PI-PLC through Mek and Erk.

egr-1 is a zinc-finger transcription factor that recognizes the sequence GCGGGGGCG, which overlaps with the Sp1 consensus sequence. It is an immediate early growth response gene induced by cytokines, certain growth factors, DNA damaging agents, and heat shock, to mention but a few. Of particular interest in the present context is its role in the regulation of inducible transcription of the TF gene (41) and its increased DNA binding upon phosphorylation (42), which may be important for its binding in preference to Sp1. This may constitute a positive feedback cycle leading to increased levels of TF when coagulation is initiated. Induction of other genes by FVIIa or FXa in addition to *egr-1* and TF has been reported, indicating that several physiological pathways may be affected. It remains to be seen what will be the physiological impact of these *in vitro* observations.

REFERENCES

1. Røttingen, J.-A., Enden, T., Camerer, E., Iversen, J.-G., and Prydz, H. (1995) *J. Biol. Chem.* **270,** 4650–4660
2. Camerer, E., Kolsto, A. B., and Prydz, H. (1996) *Thromb. Res.* **81,** 1–41
3. Poulsen, L. K., Jacobsen, N., Sørensen, B. B., Bergenhem, N. C. H., Kelly, J. D., Foster, D. C., Thastrup, O., Ezban, M., and Petersen, L. C. (1998) *J. Biol. Chem.* **273,** 6228–6232
4. Pendurthi, U. R., Alok, D., and Rao, L. V. M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 12598–12603
5. Ollivier, V., Bentolila, S., Chabbat, J., Hakim, J., and de Prost, D. (1998) *Blood* **91,** 2698–2703
6. Taniguchi, T., Kakkar, A. K., Tuddenham, E. G. D., Williamson, R. C. N., and Lemoine, N. R. (1998) *Cancer Res.* **58,** 4461–4467
7. Ott, I., Fischer, E. G., Miyagi, Y., Mueller, B. M., and Ruf, W. (1998) *J. Cell Biol.* **140,** 1241–1253
8. Randolph, G. J., Luther, T., Albrecht, S., Magdolen, V., and Muller, W. A.

(1998) *Blood* **92,** 4167–4177
9. Gajdusek, C., Carbon, S., Ross, R., Nawroth, P., and Stern, D. (1986) *J. Cell Biol.* **103,** 419–428
10. Gasic, G. P., Arenas, C. P., Gasic, T. B., and Gasic, G. J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 2317–2320
11. Ku, F. N., Yang, Y. C., Huang, S. C., and Ou, J. T. (1996) *J. Clin. Invest.* **98,** 1493–1501
12. Camerer, E., Røttingen, J.-A., Iversen, J.-G., and Prydz, H. (1996) *J. Biol. Chem.* **271,** 29034–29042
13. Schaeffer. P., Mares, A. M., Dol, F., Bono, F., and Herbert, J. M. (1997) *Circ. Res.* **81,** 824–828
14. Papapetropoulos, A., Piccardoni, P., Cirino, G., Bucci, M., Sorrentino, R., Cicala, C., Johnson, K., Zachariou, V., Sessa, W. C., and Altieri, D. C. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 4738–4742
15. Senden, N. H. M., Jeunhomme, T. M. A. A., Heenskerk, J. W. M., Wagenvoord, R., van't Veer, C., Hemker, H. C., and Buurman, W. A. (1998) *J. Immunol.* **161,** 4318–4324
16. Bono, F., Herault, J. P., Avril, C., Schaeffer, P., and Herbert, J. M. (1997) *J. Cell. Physiol.* **172,** 36–43
17. Herbert, J. M., Bono, F., Herault, J. P., Avril, C., Dol, F., Mares, A. M., and Schaeffer, P. (1998) *J. Clin. Invest.* **10,** 993–1000
18. Nicholson, A. C., Nachman, R. L., Altieri, D. C., Summers, B. D., Ruf, W., Edgington, T. S., and Hajjar, D. P. (1996) *J. Biol. Chem.* **271,** 28407–28413
19. Coughlin, S. R. (1998) *Atheroscler. Thromb. Vasc. Biol.* **18,** 514–518
20. Grand, R. J., Turnell, A. S., and Grabham, P. W. (1996) *Biochem. J.* **313,** 353–368
21. Vu, T.-K. H., Hung, D. T., Wheaton, V. I., Coughlin, S. R. C. (1991) *Cell* **64,** 1057–1068
22. Nystedt, S., Emilsson, K., Wahlestedt, C., and Sundelin, J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 9208–9212
23. Ishihara, H., Connolly, A. J., Zeng, D., Kahn, M. L., Zheng, Y. W., Timmons, C., Tram, T., and Coughlin, S. R. (1997) *Nature* **386,** 502–506
24. Kahn, M. L., Zheng, Y. W., Huang, W., Bigornia, V., Zeng, D., Moff, S., Farese, R. V., Jr., Tam, C., and Coughlin, S. R. (1998) *Nature* **394,** 690–694
25. Khachigian, L. M., and Collins, T. (1997) *Circ. Res.* **81,** 457–461
26. Boukamp, P., Petrusevska, R. T., Breitkreutz, D., Hornung, J., Markham, A., and Fuseing, N. E. (1988) *J. Cell Biol.* **106,** 761–771
27. Nystedt, S., Emilsson, K., Larsson, A.-K., Strömbeck, B., and Sundelin, J. (1995) *Eur. J. Biochem.* **232,** 84–89
28. Camerer, E., Pringle, S., Skartlien, A. H., Wiiger, M., Prydz, K., Kolstø, A.-B., and Prydz, H. (1996) *Blood* **88,** 1339–1349
29. Feinberg, A. P., and Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13
30. Feinberg, A. P., and Vogelstein, B. (1984) *Anal. Biochem.* **137,** 266–267
31. Røtnes, J. S., and Iversen, J.-G. (1992) *Cell Calcium* **13,** 487–500
32. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260,** 3440–3450
33. Røtnes, J. S, and Røttingen, J.-A. (1994) *Cell Calcium* **15,** 467–482
34. Furie, B., and Furie, B. C. (1988) *Cell* **53,** 505–518
35. Paborsky, L. R., Caras, I. W., Fisher, K. L., and Gorman, C. M. (1991) *J. Biol. Chem.* **266,** 21911–21916
36. Brass, L. F., and Molino, M. (1997) *Thromb. Haemost.* **78,** 234–241
37. Fox, M. T., Harriott, P., Walker, B., and Stone, S. R. (1997) *FEBS Lett.* **417,** 267–269
38. Bleasdale, J. E., Thakur, N. R., Gremban, R. S., Bundy, G. L., Fitzpatrick, F. A., Smith, R. J., and Bunting, S. (1990) *J. Pharmacol. Exp. Ther.* **255,** 756–768
39. Taylor, C. W., and Broad, L. M. (1998) *Trends Pharmacol. Sci.* **19,** 370–375
40. Ambrosini, G., Adida, C., and Altieri, D. C. (1997) *Nat. Med.* **3,** 917–921
41. Cui, M.-Z, Parry, G. C. N., Oeth, P, Larson, H, Smith, M, Huang, R.-P, Adamson, E. D., and Mackman, N. (1996) *J. Biol. Chem.* **271,** 2731–2739
42. Huang, R.-P., and Adamson, E. D. (1994) *Biochem. Biophys. Res. Commun.* **200,** 1271–1276

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 19, Issue of May 12, pp. 14632–14641, 2000
Printed in U.S.A.

Factor VIIa and Thrombin Induce the Expression of Cyr61 and Connective Tissue Growth Factor, Extracellular Matrix Signaling Proteins That Could Act as Possible Downstream Mediators in Factor VIIa·Tissue Factor-induced Signal Transduction*

Received for publication, January 21, 2000, and in revised form, February 23, 2000

Usha R. Pendurthi‡§, Kelly E. Allen‡, Mirella Ezban¶, and L. Vijaya Mohan Rao‖

From the Departments of ‡Molecular Biology and ‖Biochemistry, The University of Texas Health Center at Tyler, Texas 75708 and the ¶Tissue Factor/Factor VII Research, Novo Nordisk A/S Health Care Discovery, Malov DK-2760, Denmark

Extracellular interactions of plasma clotting factor VIIa (FVIIa) with tissue factor (TF) on cell surfaces trigger the intracellular signaling events. At present, it is unclear how these signals influence phenotype. To elucidate this, we have used cDNA microarray technology to examine changes in transcriptional program in human fibroblasts in response to exposure to FVIIa. cDNA microarrays revealed that FVIIa binding to TF up-regulated the expression of *Cyr61* and *CTGF* (connective tissue growth factor), the genes that encode extracellular matrix signaling proteins Cyr61 and CTGF, respectively. Northern blot analysis confirmed that FVIIa binding to TF markedly increased the expression of *Cyr61* and *CTGF* in a time- and dose-dependent manner. FVIIa catalytic activity is required for the gene induction. In addition to FVIIa, thrombin also induced the expression of *Cyr61* and *CTGF*. Hirudin abolished the thrombin-induced expression of these mRNAs but not the FVIIa-induced expression. FVIIa-induced expression of *Cyr61* appears not to involve the currently known protease-activated receptors (PARs), whereas thrombin-induced expression involves the activation of PAR1 and possibly an additional PAR. Various intracellular signaling pathway inhibitors exhibited different inhibitory pattern on FVIIa and thrombin-induced up-regulation of *Cyr61*. Cyr61 and CTGF could act as downstream mediators of FVIIa·TF in affecting various biological processes.

The primary function of plasma clotting factor VIIa (FVIIa),[1] upon its binding to TF on the cell surface, is to initiate blood coagulation (1). However, a number of recent studies suggest that FVIIa binding to TF influences an array of important biological functions other than coagulation, such as angiogenesis (2, 3), embryo vascularization (4), and tumor metastasis (2, 5, 6). At present, it is unclear how FVIIa·TF contributes to these biological processes. Recent studies have shown that FVIIa binding to TF induces various intracellular signals (7, 8). The cumulative data so far suggest that FVIIa·TF mediates cell signaling by two distinct mechanisms, TF cytoplasmic domain-dependent (independent of the proteolytic activity of FVIIa) and FVIIa·TF proteolytic activity-dependent (independent of the cytoplasmic domain of TF) (7). A growing number of recent reports suggest that the later mechanism is a predominant mechanism. For example, binding of FVIIa to cell surface TF was shown to induce intracellular Ca^{2+} oscillations in a number of TF expressing cells (9, 10), transient phosphorylation of tyrosine in monocytes (11), activation of MAP kinase (12, 13), alteration in gene expression in fibroblasts (14), up-regulation of urokinase receptor in tumor cells (15), and *Egr-1* expression in HaCaT cells (16). Because active site-inactivated factor VIIa (FVIIai) fails to induce many of the above signaling responses, from Ca^{2+} oscillations (10) to MAP kinase activation (12) and gene induction (12, 16), it appears that the catalytic activity of FVIIa is required for FVIIa·TF-mediated signal transduction. Furthermore, specific inhibitors for FXa and/or thrombin failed to inhibit the FVIIa-induced signal transduction (12, 13, 16), suggesting that the FVIIa-induced signal transduction arises directly from FVIIa proteolytic activity and not from possible generation of downstream activated coagulation factors, such as FXa and thrombin. TF cytoplasmic domain is not required for FVIIa·TF-induced proteolytic signaling because cells transfected with TF mutant lacking the cytoplasmic domain were fully capable of mediating FVIIa-induced p44/42 MAP kinase phosphorylation (13) and up-regulation of *Egr-1* (16).

At present, it is unclear how various intracellular signals induced by proteolytically active FVIIa contribute to pathophysiological functions, such as angiogenesis and tumor metastasis. Studies have shown that the FVIIa·TF complex has a strong effect on migration of cultured smooth muscle cells (17) and pancreatic cancer cells (15). The catalytic activity of FVIIa was essential for the increased cell migration in these cells. In preliminary studies, Seigbahn *et al.* (18) showed that incubation of fibroblasts with FVIIa reduces the platelet-derived growth factor concentration required to stimulate fibroblast migration by a 100-fold. Overall, all of the above data suggest that a signaling cascade involving the proteolytic function of FVIIa can result in phenotypic changes that are crucial for angiogenesis and tumor metastasis. However, so far, no clear connection has been established among the various FVIIa-induced intracellular changes. Furthermore, it is yet to be shown how FVIIa-induced intracellular changes lead to phenotypic changes. One possibility is that FVIIa could induce the expression of growth regulators that act downstream to induce cellular processes.

* This work was supported in part by a grant from the American Heart Association, Texas Affiliate (to U. R. P.) and a grant from Novo-Nordisk Health Care Discovery (to L. V. M. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Dept. of Molecular Biology, University of Texas Health Center at Tyler, 11938 U. S. Hwy. 271, Tyler, TX 75708. Tel.: 903-877-7342; Fax: 903-877-7426; E-mail: usha@uthct.edu.

[1] The abbreviations used are: FVIIa, factor VIIa; TF, tissue factor; FVIIai, active site-inactivated factor VIIa; FXa, factor Xa; CTGF, connective tissue growth factor; MAP, mitogen-activated protein; TAP, tick anticoagulant protein; PAR, protease-activated receptor.

To investigate the above possibility, in the present study, we have examined alterations in gene expression in human fibroblasts in response to FVIIa using cDNA microarray technology. Our data reveal that the cellular expression of only very few genes was detectably altered in fibroblasts upon exposure to FVIIa. Further studies confirmed that FVIIa up-regulated the expression of two related genes, *Cyr61* and *CTGF*, which are growth factor-inducible immediate early genes, the products of which were shown to promote cell adhesion, augment growth factor-induced DNA synthesis, and stimulate cell migration in fibroblasts and endothelial cells (19).

MATERIALS AND METHODS

Cell Culture—A fibroblast cell line derived from normal embryonic lung tissue (WI-38, obtained from ATCC, Manassas, VA) was grown in Dulbecco's modified Eagle's medium (GLUTAMAX with high glucose from Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (Cellgro, Herndon, VA), 1% penicillin and streptomycin (BioWhittaker, Walkersville, MD), and 1% L-glutamine (BioWhittaker). When the cells reached about 80% confluency, the serum containing growth medium was removed and the cells were washed once with Dulbecco's modified Eagle's medium and then incubated with Dulbecco's modified Eagle's medium for 20–24 h to make the cells quiescent.

Proteins—Recombinant human FVIIa, a gift from Novo Nordisk (Gentofte, Denmark), was reconstituted in sterile water at a concentration of 1–1.3 mg/ml. The stock FVIIa solutions were checked for contaminating trace levels of endotoxin using limulus amebocyte lysate (BioWhittaker), and none was detected (detection level, 30 pg). Active site-inactivated factor FVIIa was prepared by incubating recombinant human FVIIa with a peptidyl inhibitor, D-Phe-L-Phe-L-Arg chloromethyl ketone, as described earlier (20). Human factor VII was purified as described earlier (21). Factor VII preparations contained 0.03–0.4% (w/w) FVIIa in them. Human plasma FXa was purified as described previously (22) or obtained from Enzyme Research Laboratories (South Bend, IN). Thrombin was obtained from Enzyme Research Laboratories. Recombinant tick anticoagulant protein (TAP) was kindly provided by George Vlasuk (Corvas, San Diego, CA) and recombinant hirudin was obtained from either Sigma or Calbiochem (San Diego, CA). Preparation of monospecific, polyclonal rabbit anti-human TF IgG was described earlier (23). Monospecific polyclonal rabbit anti-human factor X antiserum was prepared by immunizing a rabbit with purified homogenous human factor X and IgG fractions from the antisera were obtained by precipitation with 40% ammonium sulfate saturation followed by DEAE-Affi-Gel-Blue chromatography.

cDNA Microarray—WI-38 cells were cultured to 80% confluency in T-75 flasks and serum-deprived for 24 h to cause them to enter the quiescent state as described above. The culture medium was replaced with fresh serum-free Dulbecco's modified Eagle's medium (supplemented with 5 mM $CaCl_2$) and allowed to stabilize for 2 h in tissue culture incubator. Then, the cells were treated with a control medium or the medium supplemented with recombinant FVIIa (5 μg/ml, 100 nM) for 90 min at 37 °C in a culture incubator. At the end of 90 min of treatment, total RNA was isolated from the control and FVIIa-treated cells using TRIZOL (Life Technologies, Inc.). Poly(A) RNA was purified by a double pass over Oligo Tex mRNA isolation columns as described in manufacturer's technical bulletin (Qiagen, Valencia, CA). Eight hundred ng of highly purified poly(A) RNA from the control and FVIIa-treated cells were sent for cDNA microarray analysis service (UniGEM Human V microarray, Genome Systems Inc, St. Louis, MO).

Northern Blot Analysis—Total RNA was prepared using TRIZOL reagent from quiescent monolayers of WI-38 cells that were exposed to FVIIa and other materials as described under "Results." Northern blot analysis was carried out using a standard procedure. Briefly, 10 μg of total RNA was size-fractionated by gel electrophoresis in 1% agarose/6% formaldehyde gels and transferred onto the nitrocellulose membrane by a capillary blot method. Northern blots were prehybridized at 42 °C for 2 h with a solution containing 50% formamide, 5× SSC, 50 mM Tris·HCl, pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 5 mM EDTA, 100 μg/ml denatured salmon sperm DNA, and 0.2% bovine serum albumin before hybridizing with either ^{32}P-labeled *Cyr61* or *CTGF* cDNA probes (10^6 cpm/ml) in the same solution for overnight. The hybridized membranes were exposed to either DuPont NEF or Fuji RX x-ray film. For quantification purposes, the membranes were exposed to phosphor screens for 1–4 h, and the exposed screens were analyzed in a PhosphorImager (Molecular Dynamics) using Image-Quant NT software. The units (counts) obtained in various treatments were normalized to counts present in control-treated sample and/or counts present in a positive control, such as FVIIa-treated cells.

FVIIa Binding to Cell Surface TF—^{125}I-FVIIa binding to cell surface TF was determined as described earlier (24). Briefly, confluent monolayers of WI-38 in 12-well culture plates were incubated for 2 h at 4 °C with varying concentrations of ^{125}I-FVIIa in a calcium-containing Hepes buffer (10 mM Hepes, pH 7.5, 150 mM NaCl, pH 7.5, buffer supplemented with 5 mM $CaCl_2$ and 1 mg/ml fatty acid-free bovine serum albumin). Then, unbound ^{125}I-FVIIa was removed, and the monolayers were washed four times with the buffer. The cells were removed from the well by trypsin digestion and counted for the radioactivity to determine the amount of cell-associated FVIIa. Parallel experiments were carried out in which the monolayers were first incubated for 30 min with rabbit anti-human TF IgG (100 μg/ml) before radioactive ligand was added to cells to obtain nonspecific FVIIa binding to cell surfaces. TF-specific binding was determined by subtracting the nonspecific FVIIa binding values from the corresponding binding values obtained in the absence of anti-TF antibody. In general, TF-specific binding of ^{125}I-FVIIa in WI-38 cells was in the range of 70–80%.

Factor VII Activation—Activation of factor VII bound to WI-38 cells was evaluated as described earlier (25). Briefly, monolayers of WI-38 cells were incubated at 4 °C for 90 min with ^{125}I-factor VII (10 nM) in a buffer containing benzamidine·HCl (10 mM) and calcium ions (5 mM) to allow factor VII binding to TF with minimal activation of factor VII. Then, the unbound factor VII was removed, and the monolayers were washed three times and overlaid with calcium-containing buffer. At indicated time periods, the supernatant was removed from the dish, and cell-bound ^{125}I-factor VII was eluted by incubating cells for 5 min with EDTA (5 mM). The eluates were reduced with 10% (v/v) 2-mercaptoethanol and subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography.

Chromogenic Assay—WI-38 cells were cultured in 96-well culture plates and made quiescent as described above. After washing the cells, 5 μg/ml (100 nM) FVIIa in 100 μl of calcium-containing buffer was added to the wells containing cells or to the control wells coated with buffer (no cells). After 45 min of incubation, 25 μl of chromogenic substrates for FXa or thrombin, *i.e.* Chromozym X and Chromozym TH (1.25 mM), were added to the wells and allowed to stand for 3 h to develop color. The plate was read in a microplate reader (Molecular Devices) at 405 nm. To establish the sensitivity of the assay to detect the generation of traces of FXa and thrombin, trace concentrations of factor Xa, 0.1–2 ng/well (20 pM to 0.4 nM), or thrombin, 0.2–10 milliunits/well (17 pM to 0.87 nM), were added to directly to cells cultured in parallel wells on the same plate before Chromozym X or Chromozym TH was added to the wells to develop color.

RESULTS

cDNA Microarray—The quiescent human fibroblasts (WI-38) were exposed to a control serum-free medium or the serum-free medium supplemented with recombinant human FVIIa (5 μg/ml, 100 nM) for 90 min. Poly(A) RNA was purified from these samples, and 600 ng of mRNA was labeled with either Cy3 or Cy5 fluorescence and then hybridized to the UniGem Human V chip containing 8000 sequence-verified expressed sequence tags, representing up to 5000 known human genes (service performed by Genome System Inc. for a fee). As controls, to measure sensitivity, monitor the reverse transcription reaction and purification, determine hybridization efficiency, and gain an overall view of the quality and performance of the assay, several known concentrations of reference cDNA were spiked into mRNAs during the probe generation reaction. Analysis of these controls indicated the success of hybridization process (balance coefficient, 1.69).

Global analysis of experimental data revealed minimal differences in hybridization signals between the control and FVIIa-treated samples. Only a very small number of genes showed moderate differential expression. We found up-regulation of five genes (2–3.5-fold higher in FVIIa treatment), whereas one gene was down-regulated upon FVIIa treatment (2.4-fold lower) (± 2 is a conservative estimate for determining the minimum magnitude of real ratios). One of the FVIIa up-regulated genes (2.5-fold higher in FVIIa-treated cells),

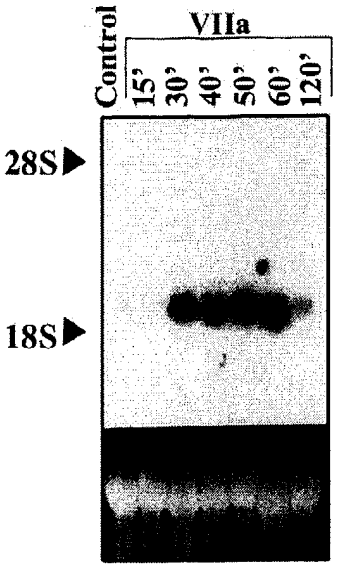


FIG. 1. **Time-dependent FVIIa-induced expression of *Cyr61*.** Quiescent monolayers of WI-38 cells were treated with FVIIa (100 nM) for varying time periods, and total RNA was harvested. Total RNA (10 μg) was subjected to Northern blot analysis and probed with radiolabeled *Cyr61* (a partial-length cDNA, obtained from Genome Systems). In this and other figures, ethidium bromide staining of 28 S or 18 S ribosomal RNA of the corresponding blot is shown in the *bottom panel* as an RNA loading control.

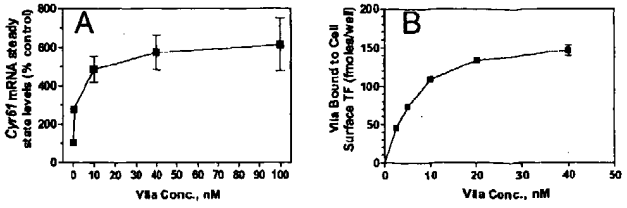


FIG. 2. **Dose-dependent FVIIa-induced expression of *Cyr61*.** *A*, quiescent monolayers of WI-38 cells in T-75 flasks were treated with varying doses of FVIIa (0, 2, 10, 40, and 100 nM) for 45 min. Total RNA (10 μg) was subjected to Northern blot analysis and probed with radiolabeled *Cyr61*. The blots were exposed to phosphor screens, and the hybridization signals were quantitated using ImageQuant NT software (n = 3). *B*, WI-38 cells cultured in 12-well plates were incubated for 2 h at 4 °C with varying concentrations of ^{125}I-FVIIa in the presence and absence of anti-TF antibodies. FVIIa associated specifically with cell surface TF was determined as described under "Materials and Methods." Data shown are mean ± S.E. (n = 4).

Cyr61, encodes an extracellular matrix signaling protein that was recently shown to promote cell proliferation, migration and tumor metastasis (19). It is interesting to note that *CTGF*, a gene belonging to the same family as *Cyr61*, was at a level 1.8-fold higher in FVIIa-treated cells than in control cells. A repeat of cDNA microarray with mRNA samples obtained in cells exposed to FVIIa for 30 min confirmed the up-regulation of both *Cyr61* and *CTGF* but not the other genes.

To validate the data obtained in microarray, we have subjected the RNA samples from the control and FVIIa-treated cells that were originally used for microarray analysis to Northern blot analysis and probed with radiolabeled *Cyr61* cDNA. The data show that *Cyr61* cDNA probe hybridized to a single transcript (approximately 2.0 kilobases) of RNA isolated from the control and FVIIa-treated cells. The hybridization signal was very faint for the RNA isolated from the control-treated cells, and the intensity of hybridization signal was higher for the RNA isolated from FVIIa-treated cells (data not shown). Quantitation of the signal revealed that the expression of *Cyr61* was 2.8-fold higher in cells exposed to FVIIa over the control-treated cells, which correlates well with the data of the microarray.

Kinetics of FVIIa-induced Expression of Cyr61—To determine the kinetics of *Cyr61* expression, quiescent fibroblasts were treated for varying time periods with 100 nM FVIIa. Total RNA was extracted and subjected to Northern blot analysis. As shown in Fig. 1, *Cyr61* expression was markedly increased in time-dependent manner in FVIIa-treated cells. The expression was peaked at about 45 min and thereafter declined close to base levels. *Cyr61* mRNA levels in cells treated with FVIIa for 45 min were 5–15-fold higher than in cells treated with a control vehicle.

To determine dose dependence of FVIIa, quiescent fibroblasts were treated with varying doses of FVIIa (0.1–5 μg/ml, 2–100 nM) for 45 min, and then total RNA samples from the cells were subjected to Northern blot analysis. As shown in Fig. 2*A*, treatment of fibroblasts with concentrations as low as 2 nM FVIIa was sufficient to induce the expression of *Cyr61*. Treatment of fibroblasts with 10 nM FVIIa, a concentration equivalent to the plasma concentration of factor VII, resulted in a prominent response, close to the maximal. The kinetics of FVIIa-induced dose-dependent expression of *Cyr61* was quite similar to the kinetics of FVIIa binding to TF receptor on WI-38 cells (Fig. 2*B*). FVIIa-induced expression of *Cyr61* is fully dependent on FVIIa binding to cell surface TF because preincubation of WI-38 cells with rabbit anti-human TF IgG (100 μg/ml) for 30 min before the addition of FVIIa completely blocked the FVIIa-induced expression of *Cyr61* (see Fig. 11).

In an additional experiment, we investigated whether a plasma concentration of factor VII is sufficient to induce the expression of *Cyr61*. Quiescent fibroblasts were treated with 10 nM factor VII for varying time periods, and the induction of *Cyr61* gene expression was evaluated by Northern blot analysis. The data show that a plasma concentration of factor VII effectively induced the expression of *Cyr61* (Fig. 3*A*). However, it requires at least 2 h of incubation with factor VII to induce the expression of *Cyr61*. This is consistent with the observation that factor VII bound to cell surface TF was slowly autoactivated to FVIIa, and a 60-min time period was required for the conversion of a substantial amount of factor VII to FVIIa (Fig. 3*B*).

Factor VIIa-induced Expression of CTGF—CTGF, a molecule structurally related to Cyr61, exhibits biological responses similar to those of Cyr61. Because the relative ratio of CTGF expression in FVIIa-treated sample *versus* the control sample in the initial cDNA microarray was 1.8, which was lower than a conservative estimate of the lowest possible real magnitude, we did not initially choose this for further examination. However, after knowing the kinetics of FVIIa-induced expression of *Cyr61* (*i.e.* FVIIa-stimulated expression of *Cyr61* reached a peak at 45 min and thereafter was repressed to base levels within 2 h after the stimulation), we thought it possible that FVIIa could also induce the expression of *CTGF* and that the minor difference in *CTGF* expression that we observed in the cDNA microarray analysis between the control and FVIIa-treated cells might be the result of a 90-min treatment period selected for the microarray assay. Therefore, we have tested the kinetics of FVIIa-induced expression of *CTGF* by Northern blot analysis. As shown in Fig. 4, FVIIa treatment markedly induced the expression of *CTGF* mRNA steady state levels in a time- and dose-dependent manner. The kinetics of FVIIa-induced expression of *CTGF* were very similar to the kinetics of FVIIa-induced expression of *Cyr61*.

Involvement of Transcriptional Mechanism in Accumulation of Cyr61 and CTGF mRNA in FVIIa-treated Fibroblasts—To

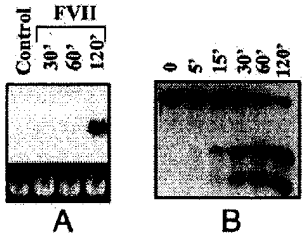


FIG. 3. **Induction of *Cyr61* expression by zymogen factor VII (*A*) correlates with autoactivation of factor VII bound to cells (*B*).** *A*, quiescent monolayers of WI-38 cells were treated with plasma factor VII (0.5 μg/ml, 10 nM) for varying time periods. Total RNA (10 μg) was subjected to Northern blot analysis and probed with radiolabeled *Cyr61*. *B* shows the extent of conversion of ^{125}I-factor VII bound to cells to ^{125}I-FVIIa at indicated time periods (see under "Materials and Methods" for the details).

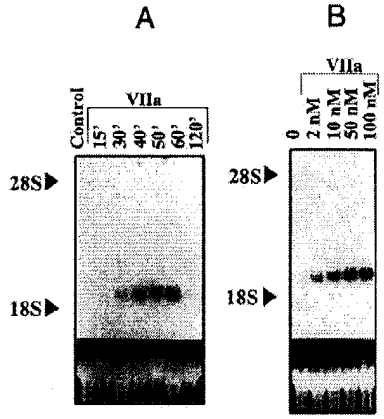


FIG. 4. **Time- and dose-dependent FVIIa-induced expression of *CTGF*.** Quiescent monolayers of WI-38 cells were treated with 100 nM FVIIa for varying time periods (*A*) or varying doses of FVIIa for 45 min (*B*). Total RNA (10 μg) was subjected to Northern blot analysis and probed with radiolabeled *CTGF*.

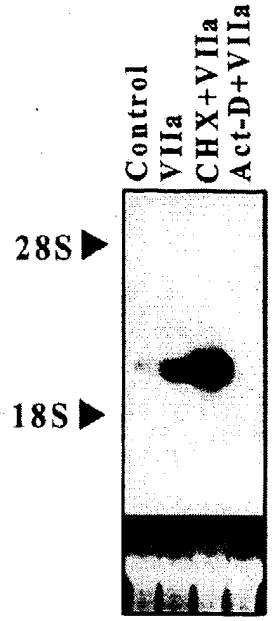


FIG. 5. **Effect of actinomycin-D and cycloheximide on FVIIa-induced *Cyr61* mRNA steady-state levels.** Quiescent monolayers of WI-38 cells were preincubated with a control vehicle, actinomycin D (*Act-D*) (10 μg/ml) or cycloheximide (*CHX*) (10 μg/ml) for 30 min before the cells were exposed to FVIIa (100 nM) for 45 min. Total RNA (10 μg) was subjected to Northern blot analysis and probed with radiolabeled *Cyr61*.

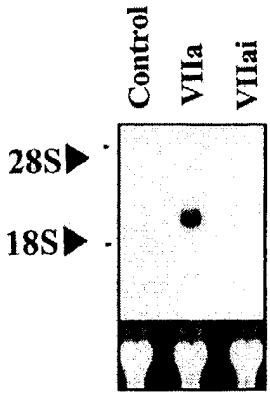


FIG. 6. **FVIIa catalytic activity is required for the induced expression of *Cyr61*.** Quiescent monolayers of WI-38 cells were treated with a control serum-free medium or serum-free medium containing FVIIa (100 nM) or FVIIai (100 nM) for 45 min. Total RNA (10 μg) was subjected to Northern blot analysis and probed with radiolabeled *Cyr61*.

investigate whether transcription is involved in FVIIa-mediated increase in *Cyr61* mRNA steady-state levels, quiescent WI-38 cells were incubated with actinomycin-D (10 μg/ml) for 30 min before the addition of FVIIa for 45 min. As shown in Fig. 5, actinomycin-D treatment completely blocked the FVIIa-induced expression of *Cyr61*. This finding indicates a transcriptional mechanism for induction of *Cyr61*. To investigate whether *de novo* protein synthesis is required for the induction of *Cyr61* mRNA by FVIIa, WI-38 cells were pretreated with a protein synthesis inhibitor, cycloheximide, before the cells were exposed to FVIIa for 45 min. As shown in Fig. 5, the stimulatory effect of FVIIa was not blocked by cycloheximide. In fact, cycloheximide markedly increased the FVIIa-induced *Cyr61* mRNA accumulation, indicating a rapid degradation of mRNA involving a labile protein, a hallmark property of immediate-early genes. Similar results were obtained with FVIIa-induced expression of *CTGF* (data not shown).

Factor VIIa Catalytic Activity Is Required for Cyr61 Induction—To test whether FVIIa catalytic activity is required for the induction of *Cyr61*, WI-38 cells were treated with FVIIa and FVIIai for 45 min and the expression of *Cyr61* was evaluated by Northern blot analysis. In contrast to FVIIa, FVIIai failed to induce the expression of *Cyr61* (Fig. 6). These data suggest that FVIIa proteolytic activity is required for the induction of *Cyr61*. In this context, it may be important to point out that FVIIai was shown to bind to cell surface TF with the same or higher affinity than FVIIa (20, 26). Furthermore, the specific inhibitors of FXa and thrombin, *i.e.* TAP and hirudin, respectively, failed to abolish the FVIIa-induced expression of *Cyr61* (Fig. 7). Quantitation of the hybridization signals from several independent experiments showed that TAP partially reduced the FVIIa-induced expression of *Cyr61*, whereas hirudin had only a minimal effect on the FVIIa-induced gene expression. Data for *Cyr61* expression were as follows (percentage normalized to basal expression found in untreated cells): FVIIa, 684 ± 94%; TAP + FVIIa, 438 ± 135%; hirudin ± FVIIa, 571 ± 109% (mean ± S.E., $n = 5$). Similar data were obtained with FVIIa-induced expression of *CTGF*. Although the difference between FVIIa-induced expression of *Cyr61* in the presence of inhibitors was not statistically significant from *Cyr61* expression in the absence of inhibitors ($p = 0.173$ for FVIIa *versus* TAP + FVIIa and $p = 0.46$, FVIIa *versus* FVIIa + hirudin), we cannot entirely discount the inhibitory effect of TAP on FVIIa-induced gene expression. At present, it is un-

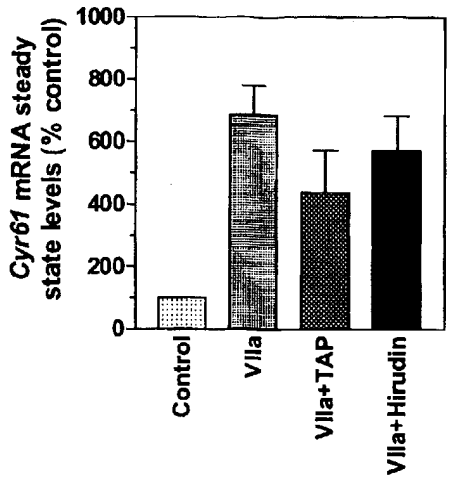


FIG. 7. **FVIIa-induced expression of *Cyr61* is not abolished by specific inhibitors of FXa and thrombin.** Quiescent monolayers of WI-38 cells were preincubated with a control medium or the medium containing 200 nM recombinant TAP or 5 units/ml hirudin for 30 min before their exposure to FVIIa (100 nM), which was preincubated correspondingly with either a control medium, TAP, or hirudin. At the end of 45 min of treatment with FVIIa, total RNA was harvested and subjected to Northern blot analysis using radiolabeled *Cyr61*. The blots were exposed to phosphor screens, and the hybridization signals were quantitated using ImageQuant NT software (n = 5, mean ± S.E.).

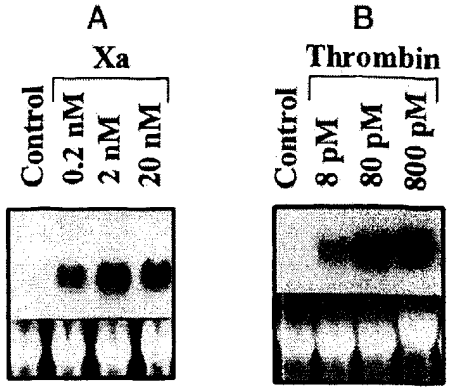


FIG. 8. **FXa and thrombin induction of *Cyr61*.** Quiescent monolayers of WI-38 cells were treated with a control medium or the medium containing varying concentrations of FXa (*A*) and thrombin (*B*) for 45 min. Total RNA (10 μg) was subjected to Northern blot analysis and probed with radiolabeled *Cyr61*.

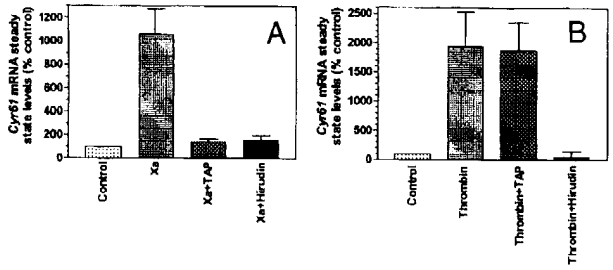


FIG. 9. **Effect of TAP and hirudin on FXa- and thrombin-induced expression of *Cyr61*.** FXa (2 nM) (*A*) and thrombin (0.1 units/ml, 0.86 nM) (*B*) were incubated with a control buffer, TAP (200 nM), or hirudin (5 units/ml) for 30 min before they were added to quiescent WI-38 cells. After 45-min treatment period, total RNA was harvested, and Northern blots were probed with radiolabeled *Cyr61* cDNA. The blots were exposed to phosphor screens, and the hybridization signals were quantitated using a PhosphorImager (mean ± S.E., n = 3 or 4 experiments).

clear the reason for this partial inhibitory effect of TAP. It is unlikely that the inhibition stemmed from a direct effect of TAP on FVIIa·TF catalytic activity because TAP, even at a concentration as high as 10 μM, had no effect on FVIIa·TF catalytic activity, whereas 200 nM TAP completely inhibited the catalytic activity of FXa (measured in amidolytic activity using 20 nM FVIIa·soluble TF complexes or FXa).

Factor Xa and Thrombin Induce the Expression of Cyr61 and CTGF—In addition to FVIIa, we also tested the effect of purified plasma FXa and thrombin for their ability to induce the expression of *Cyr61* and *CTGF*. Preliminary experiments revealed that both FXa (1 μg/ml, 20 nM) and thrombin (1 units/ml, 8.6 nM) effectively increased (10- and 25-fold, respectively) the steady state levels of both *Cyr61* and *CTGF* mRNA (data not shown). In additional experiments, we used varying concentrations of factor Xa and thrombin and found that concentrations as low as 0.2 nM FXa and 0.008 nM thrombin were sufficient to induce detectable expression of *Cyr61* (Fig. 8) and *CTGF* (data not shown).

In further experiments, we tested the effect of TAP and hirudin on FXa- and thrombin-induced expression of *Cyr61*. As shown in Fig. 9, TAP, as expected, completely inhibited the FXa-induced response. It is important to note that hirudin, a specific inhibitor of thrombin, also fully inhibited the FXa-induced response. These data raise a possibility that traces of thrombin, most probably generated on the cell surface by FXa, may be responsible for the FXa-induced response. Thrombin-induced expression of *Cyr61* is fully inhibited by hirudin and TAP had no effect on the thrombin-induced gene expression (Fig. 9).

PAR Agonist Peptide-induced Expression of Cyr61—The above data clearly establish that thrombin induces the expression of *Cyr61*. Thrombin was known to activate a number of protease-activated receptors, such as PAR1, PAR3, and PAR4. To determine whether one or more PARs are involved in the induction of *Cyr61* gene expression, first we examined whether WI-38 cells express various PARs by Northern blot analysis. These experiments revealed that WI-38 cells express mRNAs for all known PARs, *PAR1*, *PAR2*, *PAR3*, and *PAR4* (data not shown). Next, we evaluated the ability of various PAR agonist peptides to induce the expression of *Cyr61*. As shown in Fig. 10, PAR1 agonist peptide markedly enhanced, by about 10-fold, the expression of *Cyr61*. PAR2 agonist peptide substantially enhanced (4-fold increase) the expression of *Cyr61*. PAR3 and PAR4 agonist peptides have only a minimal effect on the expression of *Cyr61*. These data suggest that thrombin-induced activation of *Cyr61* gene expression, at least partly, is the result of activation of PAR1 by thrombin.

FVIIa-induced Expression of Cyr61 and CTGF Was Independent of FXa- and Thrombin-induced Response—Because our data show that both FXa and thrombin, in addition to FVIIa, would also induce the expression of *Cyr61* and *CTGF* and that the FVIIa-induced response could be partially inhibited by the specific inhibitor of FXa, TAP, it is important for us to address whether FVIIa-induced expression of *Cyr61* and *CTGF* stems from a direct action of FVIIa·TF or an indirect effect, mediated by generation of FXa and thrombin. We have used multiple independent approaches to address this.

First, we measured for possible generation of FXa and thrombin in our experimental system by using sensitive chromogenic assays. WI-38 cells were cultured in 96-well culture plates and then made quiescent using the same protocol that was used to measure FVIIa-induced expression of *Cyr61*. FVIIa (100 nM in 100 μl of calcium-containing buffer) was added to the wells containing the cells or to control wells that were coated with bovine serum albumin (no cells) and allowed to incubate for 45 min. The generation of FXa and thrombin in the

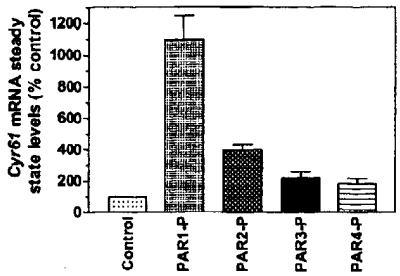


FIG. 10. **Effect of PAR agonist peptides on *Cyr61* gene expression.** Quiescent monolayers of WI-38 cells were treated with control medium or the medium containing synthetic PAR agonist peptides (25 μM) for 45 min. Total RNA (10 μg) was subjected to Northern blot analysis and probed with radiolabeled *Cyr61*. The blots were exposed to phosphor screens and the hybridization signals were quantitated using a PhosphorImager (mean ± S.E., $n = 3$). PAR agonist peptides were as follows: SFLLRN (PAR1), SLIGRL (PAR2), TFRGAP (PAR3), and GYPGQV (PAR4).

wells was monitored as described under "Materials and Methods." Minimal differences were found in the absorbance at 405 nm between the wells that contain cells ($A = 0.062$; mean of two experiments done in duplicate) and the control wells (no cells) ($A = 0.057$). These readings are at the lower end of detection limit of FXa and thrombin (20 pM), close to a blank value. In this assay, 100 pM FXa gave a reading of $A = 0.28$ and 1 milliunits/well thrombin (85 pM) gave a reading of 0.19. These data document that WI-38 cells do not generate detectable levels of FXa or thrombin endogenously upon exposure to FVIIa.

Next, we tested the effect of neutralizing anti-FX IgG on FVIIa-induced expression of *Cyr61*. As shown in Fig. 11, anti-TF IgG blocked the FVIIa-induced response, whereas anti-FX-IgG had a minimal effect on the FVIIa-induced response. As a control, anti-FX IgG was shown to effectively inhibit the FXa-induced response. Anti-TF IgG had no effect on the FXa-induced expression of *Cyr61*. These data, coupled with the above observations, confirm that FXa is not involved in the FVIIa-induced expression of *Cyr61*.

Desensitization of Transducing Receptors—The active site requirement of FVIIa to induce gene expression suggests that FVIIa induces signaling through proteolysis of a membrane-associated protein, possibly a PAR. Protease-induced PAR activation results in cleavage of the receptor and the subsequent internalization and degradation of the receptor. This makes cells refractory to a subsequent protease-induced response via the same receptor. We have used this property to test whether FVIIa-induced signaling involves the activation of the same receptor(s) that was activated by thrombin. Quiescent WI-38 cells were exposed to FVIIa or thrombin for 3 h to desensitize transducing receptors and then subsequently treated for 45 min with FVIIa, FXa or thrombin to induce the expression of *Cyr61*. As shown in Fig. 12*A*, preexposure of cells to FVIIa completely desensitized the cells to the subsequently added FVIIa but not to FXa or thrombin. In reciprocal experiments, preexposure of cells to thrombin not only desensitized the thrombin-induced response but also desensitized the FVIIa- and FXa-induced response.

Additional desensitization experiments conducted with PAR1 peptide agonist revealed that preexposure of cells to PAR1 peptide agonist did not make the cells fully refractory to the subsequent stimulus with FVIIa or thrombin. For example, FVIIa and thrombin increased the expression of *Cyr61* by 3- and 6-fold, respectively, in cells that were pretreated with PAR1 agonist peptide for 3 h (Fig. 13). However, the fold increase in *Cyr61* expression was substantially lower in PAR1 peptide pretreated cells compared with the cells that were not

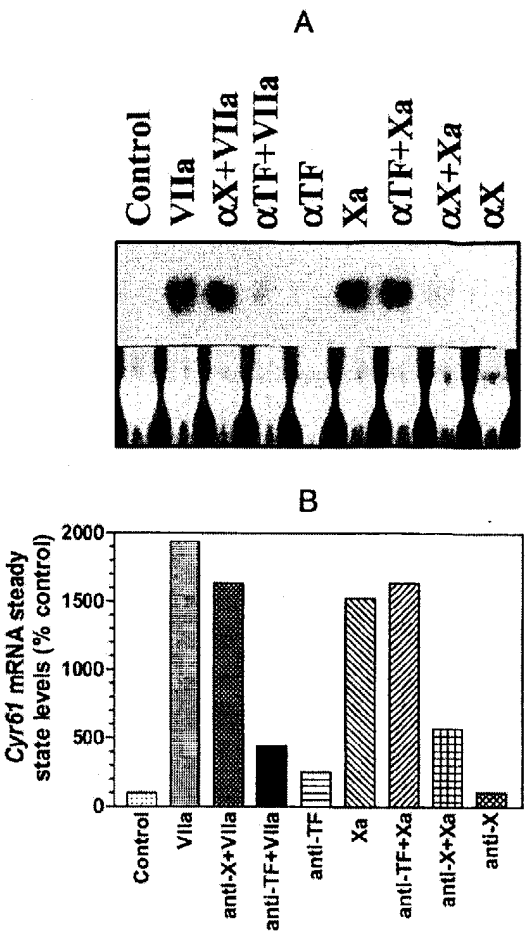


FIG. 11. **Effect of anti-TF and anti-FX IgG on FVIIa- and FXa-induced expression of *Cyr61*.** Quiescent monolayers of WI-38 cells were incubated with either control IgG, rabbit anti-human TF IgG, or rabbit anti-human FX IgG (100 μg/ml) for 30 min. Similarly, FVIIa (100 nM) and FXa (20 nM) were also incubated with antibodies before they were added to the cells. Cells were treated with FVIIa and FXa with or without antibodies for 45 min. Total RNA (10 μg) was subjected to Northern blot analysis and probed with radiolabeled *Cyr61*. *A* shows a representative autoradiograph, and *B* shows quantitative data.

pretreated with PAR1 peptide agonist. In a reciprocal experiment, preexposure of cells to FVIIa had no effect on PAR1 agonist peptide-induced expression of *Cyr61*.

In further experiments, to assess the involvement of PAR2, quiescent fibroblasts were treated with PAR2 peptide agonist (25 μM) for 3 h before they were stimulated with FVIIa for 45 min. The data show that PAR2 desensitization had no significant effect on FVIIa-induced expression of *Cyr61*. The mean data from two experiments as follow (shown as a percentage of control): PAR2 peptide for 45 min, 517%; PAR2 peptide for 3 h, 88%; PAR2 peptide for 3 h + PAR2 peptide for 45 min, 186%; PAR2 peptide for 3 h + FVIIa for 45 min, 763%; FVIIa for 45 min, 1085%.

Overall, the above desensitization studies with FVIIa, thrombin, and PAR peptide agonists suggest that FVIIa-induced expression of *Cyr61* does not involve the activation of PAR1 and PAR2 and that thrombin up-regulates the expression *Cyr61* through more than one pathway, one being the activation of PAR1.

Effect of Various Intracellular Inhibitors on FVIIa- and Thrombin-induced Up-regulation of Cyr61—To further strengthen the observation that FVIIa and thrombin up-regulate the expression of *Cyr61* through activation of different

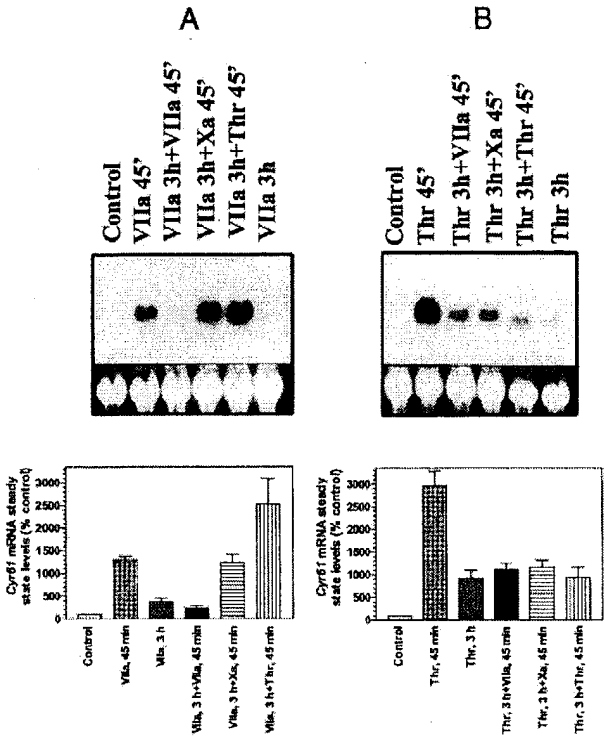


FIG. 12. **Desensitization of protease-mediated *Cyr61* gene expression.** Quiescent monolayers of WI-38 cells were treated with FVIIa (100 nM) (*A*) or thrombin (0.85 nM) (*B*) for 45 or 180 min. The monolayers that were exposed to FVIIa or thrombin for 180 min were subsequently treated for 45 min with a fresh addition of FVIIa (100 nM), FXa (20 nM), or thrombin (0.85 nM). Total RNA (10 μg) was subjected to Northern blot analysis and probed with radiolabeled *Cyr61*. The *top panels* show autoradiographs from a representative experiment, and the *bottom panels* show quantitative data (n = 4, mean ± S.E.). For quantitative purposes, *Cyr61* mRNA levels measured in quiescent cells treated with a control serum-free medium in the same experiment was taken as 100% .

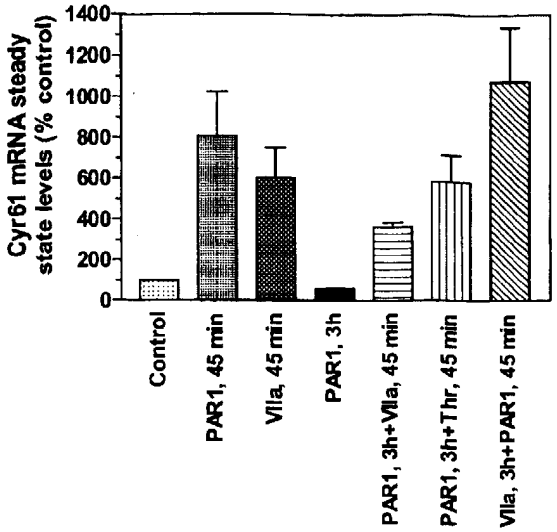


FIG. 13. **Effect of PAR1 peptide pretreatment on FVIIa- and thrombin-induced expression of *Cyr61*.** Quiescent monolayers of WI-38 cells were treated with PAR1 peptide (25 μM) for 45 min or 3 h. The cells that were treated with PAR1 peptide for 3 h were restimulated for 45 min with the subsequent addition of FVIIa (100 nM) or thrombin (0.85 nM). In parallel, cells were treated with FVIIa (100 nM) for 3 h followed by PAR1 peptide (25 μM) for 45 min. Total RNA (10 μg) was subjected to Northern blot analysis and probed with radiolabeled *Cyr61*, and hybridization signals were quantitated using a PhosphorImager (n = 4, mean ± S.E.).

signaling pathways, we have investigated the effect of various intracellular inhibitors on FVIIa- and thrombin-induced expression of *Cyr61*. As shown in Fig. 14, cholera toxin significantly inhibited both the FVIIa- and thrombin-enhanced expression of *Cyr61*, by 96 and 66%, respectively. MAP kinase inhibitor, PD 98059, markedly impaired (87% inhibition) the FVIIa-mediated increased expression of *Cyr61*, whereas it had a minimal effect (25% inhibition) on the thrombin-induced expression of *Cyr61*. Similarly, a protein tyrosine kinase inhibitor, herbimycin, and a phospholipase C inhibitor, U73122, suppressed the FVIIa-induced response by 52% and 80%, respectively, whereas they minimally inhibited (less than 25%) the thrombin-induced expression of *Cyr61*. The slight inhibition in the thrombin-induced expression of *Cyr61* observed in cells treated with PD98059, herbimycin, and U73122 was not statistically significant (p values were 0.35 or higher when compared with thrombin-induced expression of *Cyr61* in the absence of inhibitors), whereas their effect on FVIIa-induced expression was highly ($p < 0.01$ for PD 98059 and U73122) or moderately ($p = 0.07$ for herbimycin) significant. In contrast to the above inhibitors, a specific PI3-kinase inhibitor, LY 294002, markedly impaired both the FVIIa- and thrombin-induced expression of *Cyr61* (100% and 80%, respectively). These data suggest that FVIIa and thrombin, predominantly, signal through the activation of two different signaling pathways, which could merge and/or overlap downstream.

DISCUSSION

The primary function of plasma clotting FVIIa, upon its binding to TF on the cell surface, is to initiate blood coagulation. A number of recent studies suggest that FVIIa binding to TF not only triggers the coagulation cascade but also leads to other cellular processes such as angiogenesis and tumor metastasis (2). It is not yet clear how FVIIa·TF influences such complex biological processes. Although a number of recent reports (see Ref. 7) show that binding of FVIIa to TF induces intracellular signaling, it is unknown how these specific cellular events influence biological functions. One possibility is that FVIIa-induced signaling could lead to expression of growth regulators that act downstream to induce cellular processes. To investigate this possibility, in the present study, we have exposed human fibroblasts to FVIIa and examined changes in the transcriptional program using a cDNA microarray. We chose fibroblasts because these cells normally encounter serum, which contain growth factors and activated clotting factors in the context of vascular injury due to physical (*e.g.* surgery) or pathophysiological conditions. Furthermore, the temporal program of gene expression observed in response to serum suggests that fibroblasts are programmed to interpret the abrupt exposure to serum not as a general mitogenic stimulus but as a specific physiological signal (27). Characterization of transcriptional activation in response to serum and growth factors also suggests that fibroblasts are active participants in a conversation among the diverse cells that collectively control inflammation, angiogenesis, and wound healing (27).

cDNA microarray analysis with mRNA isolated from fibroblasts exposed to FVIIa for 90 min suggested a possible up-regulation of *Cyr61*. Northern blot analysis confirmed these data. In addition to *Cyr61*, the cDNA microarray also showed differential expression of four other genes, but the differential expression ratio was very close to the borderline significance, and in subsequent preliminary experiments, we could not confirm their differential expression by Northern blot analysis. In addition to *Cyr61*, FVIIa also up-regulated the expression of

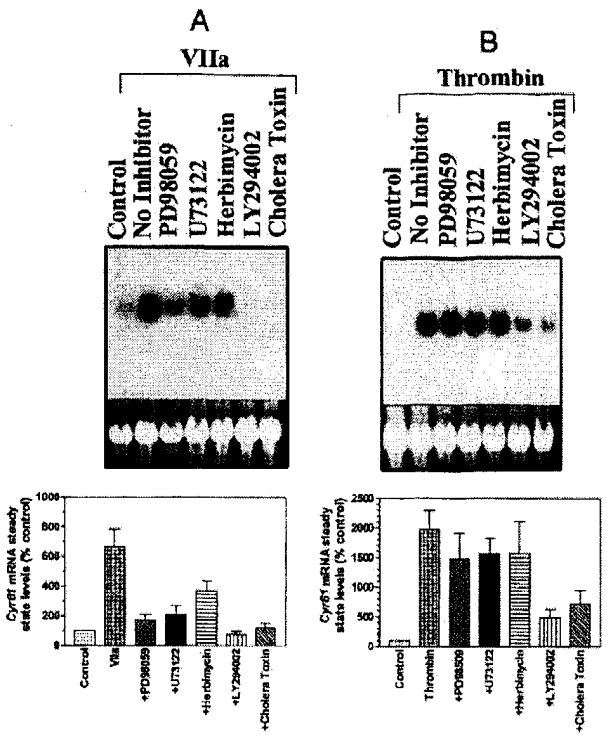


FIG. 14. **Effect of various intracellular inhibitors on FVIIa- and thrombin-induced up-regulation of *Cyr61* mRNA in WI-38 cells.** Quiescent monolayers of WI-38 cells were preincubated with inhibitors for 1 h prior to the addition of FVIIa (100 nM) (*A*) or thrombin (0.85 nM) (*B*). Total RNA was harvested 45 min after the addition of FVIIa or thrombin and subjected to Northern blot analysis using radiolabeled *Cyr61* cDNA. The concentrations of inhibitors used were as follows: PD98059, 25 μM; U73122, 50 μM; herbimycin, 5 μM; LY294002, 50 μM; and cholera toxin, 1 μg/ml. The *top panels* show autoradiographs from a representative experiment, and the *bottom panels* show quantitative data (n = 4, mean ± S.E.). For quantitative purposes, *Cyr61* mRNA levels measured in quiescent cells treated with a control serum-free medium in the same experiment was taken as 100%

CTGF, a structurally related molecule, which elicits biological responses similar to those of Cyr61. FVIIa-induced expression of *Cyr61* and *CTGF* was transient and FVIIa dose-dependent. The expression levels peaked between 30 and 60 min and thereafter reduced close to base levels in 2–3 h (Figs. 1 and 4). The kinetics of FVIIa dose-dependent expression of *Cyr61* was quite similar to the kinetics of FVIIa binding to TF. A low concentration of FVIIa (2 nM), well below the level of the corresponding circulating zymogen, is sufficient to induce the expression of *Cyr61*, and the expression levels reached a point close to maximal expression at 10 nM FVIIa (Fig. 2).

Induction of both *Cyr61* and *CTGF* expression was dependent on FVIIa binding to cell surface TF because anti-TF IgG was shown to completely block the FVIIa-induced expression of both *Cyr61* and *CTGF*. The catalytic activity of FVIIa is essential for its ability to induce the expression of *Cyr61* and *CTGF*, as evidenced by the inability of active site-inactivated FVIIa, which binds to TF with the same or a higher affinity than FVIIa (20), to up-regulate the gene expression. Although FXa and thrombin could also induce the expression of *Cyr61*, it is unlikely that they are involved in the FVIIa-induced expression of *Cyr61*. We found no evidence for the generation of traces of FXa and thrombin in our experimental system. Furthermore, hirudin, a specific inhibitor of thrombin, failed to suppress the FVIIa-induced expression of *Cyr61*. Although TAP, a specific inhibitor of FXa, partially inhibited the FVIIa-induced response, this cannot be viewed simply as FXa being responsible for the FVIIa-induced response, because all of the remaining experimental data provide support to a contrary notion. These other data are as follows: (i) no traces of FXa generation in our experimental system (detection limit, 20 pM, a concentration well below the concentration of FXa required to induce *Cyr61*); (ii) neutralizing anti-FX antibodies failed to suppress the VIIa-induced response, whereas they effectively blocked the FXa-induced response (Fig. 11); and (iii) hirudin completely suppressed the FXa-induced response (Fig. 9), whereas it had no significant effect on the FVIIa-induced response (Fig. 7). These data suggest that FVIIa-induced expression of *Cyr61* is independent of FXa and that the FXa-induced expression of *Cyr61* might have stemmed from generation of undetectable levels of thrombin in our experimental system. In this context, it may be pertinent to point out that FXa used in present study, although highly purified and apparently homogenous, is derived from plasma and as low as 8 pM thrombin (which is below our detection limit) is sufficient to induce the expression of *Cyr61*.

The observation that FVIIa proteolytic activity is required for FVIIa-induced expression of *Cyr61* and *CTGF* suggests two possible mechanisms by which FVIIa could act. One possibility is that FVIIa could release, by its proteolytic action, a growth factor-like substance (which might have been sequestered on the cell surface or the extracellular matrix) that directly links to signal transducing receptor system. Factor Xa (28) and thrombin (29) have been shown to cause an autocrine release/expression of growth factors. However, this possibility is unlikely because exposure of cells that were preincubated with anti-TF IgG (to block the direct action of FVIIa) to FVIIa conditioned medium did not lead to up-regulation of *Cyr61* expression (data not shown). Rapid induction of *Cyr61* by FVIIa precludes the possible involvement of *de novo* synthesized growth factors in FVIIa-induced response. Furthermore, cycloheximide, the protein synthesis inhibitor, failed to block the FVIIa-induced expression of both *Cyr61* and *CTGF*. These data are consistent with our recent observation that FVIIa-conditioned medium failed to induce MAP kinase activation in baby hamster kidney cells transfected with TF (30).

A second and more likely possibility is that FVIIa proteolytically activates a cell surface receptor, akin to a PAR. Earlier studies, using Ca^{2+} release (10, 30), up-regulation of *Egr-1* mRNA (16), and MAP kinase activation (13) as markers for FVIIa-induced signaling, suggested this possibility. However, a direct evidence is lacking for this. So far, four PARs (PAR1, PAR2, PAR3, and PAR4) have been identified. Northern blot analysis of RNA isolated from WI-38 cells with various PAR-specific cDNA probes demonstrated that WI-38 cells express all known PARs (data not shown). Therefore, all four known PARs are potential candidate substrates for FVIIa. Thrombin activates PAR1, PAR3, and PAR4 and thus is likely to desensitize these receptors (31). Pretreatment of the cells with thrombin down-regulated the ability of FVIIa to induce the expression of *Cyr61*, raising the possibility that FVIIa may be activating one of the thrombin-activable PARs. Experiments with PAR-specific peptide agonists revealed that PAR1 and PAR2 peptide agonists, and not other peptide agonists, substantially up-regulated the expression of *Cyr61* (Fig. 10) and *CTGF* (data not shown). However, unlike thrombin, PAR1 peptide agonist pretreatment did not fully abolish the FVIIa-induced response. Similarly, desensitization with PAR2 peptide agonist had no substantial effect on the FVIIa-induced response. The cumulative data of these experiments suggest, although they do not prove, that FVIIa-induced expression of *Cyr61* does not involve any known PARs. Although such a conclusion is in full agreement with the conclusion reached in recent studies (16, 30), our

data differ from the data of these studies in one main aspect. Earlier studies, which used different cell types, showed that receptors down regulated by thrombin were not involved in FVIIa-induced signaling events (16, 30), whereas the present data show that thrombin treatment down regulated the FVIIa-induced response. A possible explanation for these differences could be that FVIIa may be activating more than one type of PAR, the expression of which is cell-specific. Alternatively, in our experiments, thrombin desensitized the FVIIa-induced response not because they share a common receptor but because the thrombin-induced signal pathways consumed one or more of downstream mediators that are necessary for the FVIIa-induced response. The latter possibility may also explain why PAR1 peptide pretreatment partially reduced the FVIIa-induced response. The observation that PAR1 peptide pretreatment did not completely abolish the thrombin-induced expression of *Cyr61* suggests that thrombin may also up-regulate the expression of *Cyr61* via an additional pathway.

Induction of *Cyr61* by both FVIIa and thrombin was abrogated by cholera toxin, which supports the involvement of G-protein, Gs, in FVIIa and thrombin-mediated signal transduction in WI-38 cells. PLC-inhibitor U73122 and MAP kinase inhibitor PD98059 markedly suppressed the FVIIa-induced expression of *Cyr61* but had only minimal effect on thrombin-induced expression of *Cyr61*. These data suggest that FVIIa induces the expression of *Cyr61* via PLC through the activation of MAP kinase. These observations are consistent with the earlier observations made with FVIIa-induced expression of *Egr-1* (16) and the fact that FVIIa-induces the activation of MAP kinase in many cell types expressing TF (12). LY 294002, the specific PI 3-kinase inhibitor, inhibited both the FVIIa- and thrombin-induced response, indicating that the second messenger, IP3, is a common mediator of the two responses. The differential effect of various cellular inhibitors on FVIIa- and thrombin-induced up-regulation of *Cyr61* further strengthens our conclusion that FVIIa-induced up-regulation of *Cyr61* does not involve the action of thrombin.

At present, it is unknown whether TF cytoplasmic tail plays any role in FVIIa-induced expression of *Cyr61* and *CTGF*. Experiments conducted with baby hamster kidney cells transfected with full-length TF and cytoplasmic tail deleted TF (13) did not provide any meaningful data. Human *Cyr61* cDNA probe hybridized to multiple RNA species in both control and FVIIa-treated baby hamster kidney cells. However, based on earlier studies, which showed FVIIa-induced activation of MAP kinase (13) and *Egr-1* induction (16) were independent of TF intracellular domain, it seems unlikely that TF cytoplasmic tail plays a role in FVIIa-induced gene expression in fibroblasts. Although TF cytoplasmic domain was shown to be required in mediating some specific cellular events (32, 33), it should be noted that these events were independent of FVIIa.

Cyr61 is an immediate-early gene that is transcriptionally activated by serum growth factors in fibroblasts (34). It encodes a secreted 40-kDa, cysteine-rich and heparin-binding protein that associates with extracellular matrix and cell surfaces (35). *Cyr61* is a member of an emerging gene family of conserved and modular proteins characterized by the presence of an N-terminal secretory signal, followed by four modular structural domains and 38 cysteine residues that are largely conserved among members of the family (19). The Cyr61 protein is shown to (i) promote the attachment and spreading of endothelial cells in a manner similar to that of fibronectin, (ii) enhance the effects of basic fibroblast growth factor and platelet-derived growth factor on the rate of DNA synthesis of fibroblasts and vascular endothelial cells, and (iii) promote cell migration in both fibroblasts and endothelial cells (36). Recent studies show that Cyr61 acts as a ligand to integrin $\alpha_v\beta_3$ (37), an adhesion receptor known to be involved in signaling that regulates a number of cellular processes including angiogenesis and tumor metastasis (38, 39). Expression of *Cyr61* in tumor cells was shown to promote tumor growth and vascularization (40). Thus, it is possible that the FVIIa-induced expression of Cyr61, acting through integrin $\alpha_v\beta_3$, may play an important role as a downstream mediator in mediating FVIIa·TF-induced effect, such as promotion of angiogenesis and tumor metastasis. The observations that FVIIa catalytic activity is required for migration of various cell types (15, 17, 18) and tumor metastasis (6, 41) are consistent with the requirement of FVIIa catalytic activity for the induction of *Cyr61*.

Although CTGF behaves very similar to Cyr61, subtle differences exists between them (see Refs. 19 and 42). For example, (i) CTGF has shown to be mitogenic in itself, whereas Cyr61 has no intrinsic mitogenic activity but augments growth factor-induced DNA synthesis; (ii) Cyr61 stimulates chemotaxis, whereas CTGF stimulates both chemotaxis and chemokinesis; and (iii) although both Cyr61 and CTGF are extracellular matrix-associated signaling molecules, CTGF is shown to secrete into culture medium (43). Thus, it is possible that FVIIa could regulate cellular functions locally via Cyr61, whereas FVIIa acts at a distance from its site through the secretion of CTGF.

It is interesting to note that, similar to TF expression (44–46), *CTGF* mRNA is undetectable in normal blood vessels but overexpressed in atherosclerotic lesions (47). In atherosclerosis, high levels of CTGF expression may be responsible for extracellular matrix accumulation and thus progression of atherosclerotic lesions (48). It is possible that overexpression of CTGF in atherosclerotic plaque could have been the result of increased expression of TF in atherosclerotic plaque. Activation of TF-mediated coagulation pathway not only plays a major role in determining plaque thrombogenicity but may also have other effects on the vessel wall (49). For example, thrombin (50, 51) and FXa (52), the intermediatory byproducts FVIIa·TF coagulation pathway, are shown to promote vascular smooth cell proliferation and thus may play a role in the development of intimal hyperplasia. More importantly, recent studies suggest that FVIIa·TF may have direct effect on aortic smooth cell migration and neonatal aortic smooth cell proliferation (17, 53, 54). It is possible that some, if not all, of these events are mediated by FVIIa·TF-induced expression of CTGF. If so, CTGF may represent the downstream effector for FVIIa·TF. Currently, we are in the process of developing the necessary reagents (recombinant Cyr61 and CTGF proteins and antibodies against them) to test the above possibilities.

REFERENCES

1. Rapaport, S. I., and Rao, L. V. M. (1995) *Thromb. Haemostasis* **74,** 7–17
2. Ruf, W., and Mueller, B. M. (1996) *Curr. Opin. Hematol.* **3,** 379–384
3. Shoji, M., Hancock, W. W., Abe, K., Micko, C., Casper, K. A., Baine, R. M., Wilcox, J. N., Danave, I., Dillehay, D. L., Matthews, E., Contrino, J., Morrissey, J. H., Gordon, S., Edgington, T. S., Kudryk, B., Kreutzer, D. L., and Rickles, F. R. (1998) *Am. J. Pathol.* **152,** 399–411
4. Carmeliet, P., Mackman, N., Moons, L., Luther, T., Gressens, P., van Vlaenderen, I., Demunck, H., Kasper, M., Breier, G., Evrard, P., Muller, M., Risau, W., Edgington, T., and Collen, D. (1996) *Nature* **383,** 73–75
5. Bromberg, M. E., Konigsberg, W. H., Madison, J. F., Pawashe, A., and Garen, A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 8205–8209
6. Mueller, B. M., and Ruf, W. (1998) *J. Clin. Invest.* **101,** 1372–1378
7. Ruf, W., and Mueller, B. M. (1999) *Thromb. Haemostasis* **82,** 175–182
8. Prydz, H. S., Camerer, E., Rottingen, J., Wiiger, M. T., and Gjernes, E. (1999) *Thromb. Haemostasis* **82,** 183–192
9. Rottingen, J. A., Enden, T., Camerer, E., Iversen, J. G., and Prydz, H. (1995) *J. Biol. Chem.* **270,** 4650–4660
10. Camerer, E., Rottingen, J. A., Iversen, J. G., and Prydz, H. (1996) *J. Biol. Chem.* **271,** 29034–29042
11. Masuda, M., Nakamura, S., Murakami, T., Komiyama, Y., and Takahashi, H. (1996) *Eur. J. Immunol.* **26,** 2529–2532
12. Poulsen, L. K., Jacobsen, N., Sorensen, B. B., Bergenhem, N. C. H., Kelly, J. D., Foster, D. C., Thastrup, O., Ezban, M., and Petersen, L. C. (1998) *J. Biol. Chem.* **273,** 6228–6232
13. Sorensen, B. B., Freskgard, P., Nielsen, L. S., Rao, L. V. M., Ezban, M., and

Petersen, L. C. (1999) *J. Biol. Chem.* **274,** 21349–21354
14. Pendurthi, U. R., Alok, D., and Rao, L. V. M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 12598–12603
15. Taniguchi, T., Kakkar, A. K., Tuddenham, E., Williamson, R., and Lemoine, N. R. (1998) *Cancer Res.* **58,** 4461–4467
16. Camerer, E., Rottingen, J., Gjernes, E., Larsen, K., Skartlien, A. H., Iversen, J., and Prydz, H. (1999) *J. Biol. Chem.* **274,** 32225–32233
17. Sato, Y., Asada, Y., Marutsuka, K., Hatakeyama, K., Kamikubo, Y., and Sumiyoshi, A. (1997) *Thromb. Haemostasis* **78,** 1138–1141
18. Siegbahn, A., Johnell, M., Rorsman, C., Heldin, C., and Ronnstrand, L. (1999) *Thromb. Haemostasis* (suppl.), 505
19. Lau, L. F., and Lam, S. (1999) *Exp. Cell Res.* **248,** 44–57
20. Sorensen, B. B., Persson, E., Freskgard, P., Kjalke, M., Ezban, M., Williams, T., and Rao, L. V. M. (1997) *J. Biol. Chem.* **272,** 11863–11868
21. Rao, L. V. M., and Bajaj, S. P. (1984) *Anal. Biochem.* **136,** 357–361
22. Rao, L. V. M., Robinson, T., and Hoang, A. D. (1992) *Thromb. Haemostasis* **67,** 654–659
23. Rao, L. V. M. (1988) *Thromb. Res.* **51,** 373–384
24. Iakhiaev, A., Pendurthi, U. R., Voigt, J., Ezban, M., and Rao, L. V. M. (1999) *J. Biol. Chem.* **274,** 36995–37003
25. Rao, L. V. M., Williams, T., and Rapaport, S. I. (1996) *Blood* **87,** 3738–3748
26. Sorensen, B. B., and Rao, L. V. M. (1998) *Blood Coagul. Fibrinolysis* **9** (suppl.), S67–S71
27. Iyer, V., Eisen, M. B., Ross, D. T., Schuler, G., Moore, T., Lee, J., Trent, J. M., Staudt, L. M., Hudson, J., Boguski, M. S., Lashkari, D., Shalon, D., Botstein, D., and Brown, P. O. (1999) *Science* **283,** 83–87
28. Ko, F. N., Yang, Y. C., Huang, S. C., and Ou, J. T. (1996) *J. Clin. Invest.* **98,** 1493–1501
29. Molloy, C. J., Pawlowski, J. E., Taylor, D. S., Turner, C. E., Weber, H., and Peluso, M. (1996) *J. Clin. Invest.* **97,** 1173–1183
30. Petersen, L. C., Thastrup, O., Hagel, G., Sorensen, B. B., Freskgard, P., Rao, L. V. M., and Ezban, M. (2000) *Thromb. Haemostasis,* in press
31. Coughlin, S. R. (1999) *Thromb. Haemostasis* **82,** 353–356
32. Ott, I., Fischer, E. G., Miyagi, Y., Mueller, B. M., and Ruf, W. (1998) *J. Cell Biol.* **140,** 1241–1253
33. Abe, K., Shoji, M., Chen, J., Bierhaus, A., Danave, I., Micko, C., Casper, K., Dillehay, D. L., Nawroth, P., and Rickles, F. R. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 8663–8668
34. O'Brien, T. P., Yang, G. P., Sanders, L., and Lau, L. F. (1990) *Mol. Cell. Biol.* **10,** 3569–3577
35. Yang, G. P., and Lau, L. F. (1991) *Cell Growth Differ.* **2,** 351–357
36. Kireeva, M. L., Mo, F., Yang, G. P., and Lau, L. F. (1996) *Mol. Cell. Biol.* **16,** 1326–1334
37. Kireeva, M. L., Lam, S., and Lau, L. F. (1998) *J. Biol. Chem.* **273,** 3090–3096
38. Hynes, R. O. (1992) *Cell* **69,** 11–25
39. Eliceiri, B. P., and Cheresh, D. A. (1999) *J. Clin. Invest.* **103,** 1227–1230
40. Babic, A. M., Kireeva, M. L., Kolesnikova, T. V., and Lau, L. F. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 6355–6360
41. Bromberg, M. E., Sundaram, R., Homer, R. J., Garen, A., and Konigsberg, W. H. (1999) *Thromb. Haemostasis* **82,** 88–92
42. Grotendorst, G. R. (1997) *Cytokine Growth Factor Rev.* **8,** 171–179
43. Kireeva, M. L., Latinkic, B. V., Kolesnikova, T. V., Chen, C., Yang, G. P., Abler, A. S., and Lau, L. F. (1997) *Exp. Cell Res.* **233,** 63–77
44. Wilcox, J. N., Smith, K. M., Schwartz, S. M., and Gordon, D. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 2839–2843
45. Marmur, J. D., Thiruvikraman, S. V., Fyfe, B. S., Guha, A., Sharma, S. K., Ambrose, J. A., Fallon, J. T., Nemerson, Y., and Taubman, M. B. (1996) *Circulation* **94,** 1226–1232
46. Thiruvikraman, S. V., Guha, A., Roboz, J., Taubman, M. B., Nemerson, Y., and Fallon, J. T. (1996) *Lab. Invest.* **75,** 451–461
47. Oemar, B. S., Werner, A., Garnier, J. M., Do, D. D., Godoy, N., Nauck, M., Marz, W., Rupp, J., Pech, M., and Luscher, T. F. (1997) *Circulation* **95,** 831–839
48. Oemar, B. S., and Luscher, T. F. (1997) *Arterioscler. Thromb. Vasc. Biol.* **17,** 1483–1489
49. Taubman, M. B., Fallon, J. T., Schecter, A. D., Giesen, P., Mendlowitz, M., Fyfe, B. S., Marmur, J. D., and Nemerson, Y. (1997) *Thromb. Haemostasis* **78,** 200–204
50. Huang, C. L., and Ives, H. E. (1987) *Nature* **329,** 849–850
51. McNamara, C. A., Sarenbock, I. J., Gimple, L. W., Fenton II, J. W., Coughlin, S. R., and Owens, G. K. (1993) *J. Clin. Invest.* **91,** 94–98
52. Gasic, G. P., Arenas, C. P., Gasic, T. B., and Gasic, G. J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 2317–2320
53. Sato, Y., Asada, Y., Marutsuka, K., and Hatakeyama, K. (1996) *Thromb. Haemostasis* **75,** 389–392
54. Kamikubo, Y., Nakahara, Y., Takemoto, S., Hamuro, T., Miyamoto, S., and Funatsu, A. (1997) *FEBS Lett.* **407,** 116–120